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OF

BARTONELLA BACILLIFORMIS.

from

~~Department of Microbiology~~
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Morgantown, West Virginia

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*In conducting the research reported herein, the investigator(s) adhered to 'Guide for Laboratory Animal Facilities and Care' established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, NAS-NRC.

INTRODUCTION

The investigations to be described were initiated on January 1, 1963 and terminated on April 30, 1966. The work was supported by Contract DA-18-064-AMC-78(A) from the United States Army Biological Laboratories.

To our knowledge, this was the only investigation being carried out in the United States concerning any facet directly related to *B. bacilliformis*. Due to this aspect, we relied on the available literature and correspondence as guides in our early studies. However, certain difficulties were encountered, particularly in the process of developing rapid and efficient methods of cultivation and identification, which necessitated our obtaining first-hand information from Peruvian investigators. Such information, based on our personal experiences in Peru, enabled us to pursue our investigations from the standpoint of validity and will be discussed relative to our investigations in the Discussion portion of the paper.

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ABSTRACT

The genus Bartonella was re-evaluated in terms of characterization and identification. Such aspects were based on serological procedures (FA, immuno-diffusion and hemagglutination techniques) and morphological, biochemical and cultural studies. The results obtained explained the lack of universally accepted properties of the organism and provided a means with which to pursue additional investigations from the standpoint of validity.

Investigations in direct concern of the pathogenicity of B. bacilliformis were directed towards reproducing the various forms of Bartonellosis in various species of animals and/or to determine their relative susceptibilities or resistances to the organism. The rabbit was observed to be more susceptible than the Rhesus monkey, guinea pig, suckling mouse or hamster when employing the intravenous, subcutaneous, intraperitoneal or intratesticular route of inoculation. A distinct alteration of the rabbits normally operative hemopoetic system could be induced. However, a distinct anemia and/or an asymptomatic infectious process could not be induced, presumably due to the comparatively high, specific resistance of the animal. Such findings were substantiated by the fact that splenectomy or the use of agents known to decrease normal host resistance had no promoting effect. Dermal lesions were produced in sensitive animals at the site of the intradermal inoculations. However, such lesions could not be produced in non-immunized rabbits. When the intratesticular route of inoculation was employed, an orchitis of severe degree was produced.

Boiled cell suspensions of the organism were found capable of sensitizing the rabbit to such a degree that anaphylactic shock could be provoked. This evidence led to the isolation of an endotoxic component from B. bacilliformis and subsequent experimental evidence indicated that this metabolite was responsible for the pathological effects observed in the rabbit. To elucidate these findings a series of immuno-pathogenicity studies were performed. It was observed that sensitization with viable cells of B. bacilliformis increased the susceptibility of rabbits to the lethality of subsequently administered Bartonella metabolites. A distinct immunological basis for the observed lethality was established and discussed in relationship to human Bartonellosis.

INTRODUCTION

The investigations to be described were initiated on January 1, 1963 and terminated on April 30, 1966. The work was supported by Contract DA-18-064-AMC-78(A) from the United States Army Biological Laboratories.

Outlined below are the main, broad objectives and procedures of the investigation in direct concern to studies on the pathogenicity and related characteristics of *Bartonella bacilliformis:*

A. Attempt to increase pathogenicity
   1. Re-evaluate methods of cultivation and nutrition in relation to pathogenicity.

   2. Determine the susceptibilities and resistances of certain animal species.

B. Test for pathogenicity employing various animal species.

C. Apply various routes and modes of inoculation.

D. Determine methods of standardizing doses.

   1. Establish methods of stabilization.

   2. Carry out associated tests of viability and stability.

E. Develop methods that would facilitate rapid identification in tissue and in culture.

F. Elucidation of the pathogenicity of *B. bacilliformis* through immunological studies.

G. Develop methods that would facilitate characterization and identification.

To our knowledge, this was the only investigation being carried out in the United States concerning any facet directly related to *B. bacilliformis*. Due to this aspect, we relied on the available literature and correspondence as guides in our early studies. However, certain difficulties were encountered, particularly in the process of developing rapid and efficient methods of cultivation and identification, which necessitated our obtaining first-hand information from Peruvian investigators. Such information, based on our personal experiences in Peru, enabled us to pursue our investigations from the standpoint of validity and will be discussed relative to our investigations in the Discussion portion of the paper.

REVIEW OF THE LITERATURE

I. History

Bartonella bacilliformis is the etiological agent of Bartonellosis (70, 83,84) which is principally endemic to certain zones of South America and Central America (40,43,45,21,22,23,18,24). This disease is not known to occur spontaneously in other parts of the world. Human Bartonellosis is differentiated, on the basis of clinical syndromes, into two conditions: Oroya Fever and Verruga Peruana. *Bartonella bacilliformis* is capable of invading the red blood cells, causing a severe anemia (Oroya Fever) and also, of multiplying in fixed tissue cells, causing a skin eruption (Verruga Peruana) (67,41,131).

Oroya Fever and Verruga Peruana, or Bartonellosis (for purposes to be considered as an infection with *Bartonella bacilliformis*), were for many years assumed to be two entirely different diseases; not to be simply varying clinical manifestations of one etiological disease.

Evidence antedating the introduction of writing in Peru suggests that Bartonellosis has existed for many years in South America. This evidence is partially archeological and partially linguistic (130). Excavated pottery, attributable to the Chimu people who flourished before the Inca conquest, depicts pathological conditions. Among these representations of diseased states, Verruga Peruana has been identified (130). Over four centuries ago, during the Inca reign, thousands are said to have died from this malady (107).

Written references to Verruga Peruana begin with the Spanish during their conquest of Peru in the 16th century. The earliest scriptural record referrable to Verruga Peruana is in 1531 when a quarter of Pizzaro's Army were reported to have perished from the disease (130,107).

The anemic form attracted little writing until the latter part of the 19th century. At this time medical attention became focused on the disease when an epidemic outbreak occurred at the construction site of the Central Railway between Lima and Oroya (130). Thus, the name Oroya Fever was given to the disease. At this time, little was known concerning the nature of the disease(s) and the bond between Oroya Fever and Verruga Peruana had not as yet received much consideration.

In 1885, Daniel Carrion, a medical student, was inoculated with material from a verrugous lesion (136). He subsequently developed Oroya Fever and ultimately succumbed to it. This martyrdom gave much impetus to the concept of the unitarian etiology of Bartonellosis with the eponym "Carrion's Disease" replacing the term Oroya Fever, honoring Daniel Carrion. With the later, cultural developments of Noguchi and co-workers in 1926, the unitarian view became established (67). Thus, history indicates the long period required to establish the etiology and unify the syndromes of Oroya Fever and Verruga Peruana.

II. Clinical Manifestations.

References 130, 95 and 98 treat the subject of the clinical manifestations of Bartonellosis in a review style and form the basis of this section. Bartonellosis is basically characterized by two, well defined clinical conditions, Oroya Fever and Verruga Peruana. However, the infection may be sub-clinical and asymptomatic (41).

Following a variable incubation period of 17-21 days, or up to 60 days (126,9,94), the initial symptoms of mild intermittant fever (37.5°-38.5°C.), pain of variable intensity in the bones, joints and muscles, along with cramps, and general malise present themselves. These initial symtoms may be accompanied by chills with intermittent higher fever, copius sweating, headaches and occasionally delerium. From this rather insidious onset the patient develops one of the three classical types of Bartonellosis. These syndromes will be described separately for clarity and convenience.

A. Hematic Syndrome (Oroya Fever)

The physical examination of the patient with Oroya Fever is not particularly revealing but the most characteristic finding is generalized lymphadenopathy with nontender nodes without pariadenitis. The spleen may or may not be enlarged except in cases complicated by an intercurrent infection (130,96,97,98). The liver is often moderately enlarged. The patient may have a discoloration of the skin and sclerae due to a slightly elevated serum bilirubin in combination with the pallor of an extreme anemia.

There is a varying degree of apathy, headache, vertigo, restlessness and drowsiness, tinnitus, exhaustion, insomnia and occasionally angina pectoris. All are dependent on the intensity of the anemia and resultant from it. Thirst and anorexia are common complaints. An upper respiratory infection may be simulated by the development of a cough and expectoration with ronchi and rales.

The cardiovascular signs which may or may not be present are: moderately hypotensive blood pressure, tachycardia and soft hemic murmurs of varied intensity heard over the entire precardium. Synchronously, there are seprasternal, epigastric and carotid pulsations, a common complaint being that the patient is hearing his heartbeat in his ears.

A hemmorhagic tendency may be manifested by petechial spots to ecchymoses, epistaxis, ginginal hemorrhage, hematemesis or melena. The most common are petechial spots (thrombocytopenic purpura).

Examination of the peripheral blood is very revealing and characteristic. There is always a degree of anemia which rapidly becomes more severe. In fact, is comparable only to severe hemorrhagic anemia in development. The anemia is macrocytic and hypochromic, the Color Index being greater than 1.0 (hemoglobin less reduced than the red blood cell count), the Mean Corpuscular Hemoglobin Concentration is low (a greater decrease in the hemoglobin content than in the volume of packed cells), as a result of the quantity of hemoglobin in the red blood cells in proportion to their size.

Marked regenerative activity is indicated in both erythroid and myeloid series. The number of nucleated red blood cells may be exceedingly high and reticulocytes may increase to 50% in severe cases (7). Circulating myeloblasts, myelocytes and metamyelocytes may be present. The erythrocytes often show basophilic stippling, Howell-Jolly bodies, Cabot rings, polychromatophilia, anisocytosis and poikilocytosis.

The pathognomonic sign of Oroya Fever is the presence of _B. bacilliformis_ in, or on, the erythrocyte. Up to 90% of the red blood cells may be infected in a severe infection. When peripheral blood films are stained with Giemsa or Leishman stains, the organism appears as a red-violet rod, situated in, or on (at present a point of controversy) the red blood cells. The organisms may occur singly, distributed in X, V, or Y forms or in appearance as chains. They may also be curved and show polar enlargement (29,134). In intense infections, the organism may also be observed within the circulating monocytes. Preceding the appearance of the organisms in the peripheral blood, they have been observed within cells of the bone marrow.

The white blood cell count is variable, a slight leukocytosis being common, but the count may be leukopenic or normal. There is frequently a "left shift" (presence of myeloblasts, myelocytes and metamyelocytes in the peripheral blood) noted upon differential white blood cell examination.

As the hematic infection runs its course, or the patient is treated with antibiotics, the organism gradually changes from a rod to a coccoid form. Shortly following this morphological transformation, the organism suddenly disappears from the peripheral blood and subsequently the patient appears to convalesce. The red blood cell count begins to rise, there is a reversion towards normal serum bilirubin and a reversion towards a normal white blood cell count is indicated by a "right shift" in the myeloid series. This period of transition in which the organism disappears and the patient appears to be convalescing is often referred to as the "critical stage" (95,96,58). Clinical improvement may not parallel the disappearance of _B. bacilliformis_ from the blood as the patient at this time appears to be predisposed to intercurrent infections, usually by organisms of the gut (96,28).

The microscopic histologic examination of tissues taken at autopsy shows intracytoplasmic development of the organism within the lining cells of the blood and lymph capillaries. This development often reaches an extreme degree, causing the cytoplasm to bulge into the lumen of the vessel. The infected cells are classified as phagocytic endothelium and resemble cells during extreme phagocytosis following blockade of the reticuloendothelium. The organisms appear as granules or clumps of granules often filling the entire cytoplasmic area, or they may appear as discrete rod forms (107,83,84,54,55,71). The infected cells do not show uniform distribution of organisms; the tendency of the organism being to form clumps and rounded masses. The nuclei are at times obscured or distorted due to the masses of the organism. However, the organism have not been demonstrated in a distinctly intranuclear position. In addition to the infection of the phagocytic endothelium, heavy infection has been noted in the capillaries of the cortical region of the kidney. The lymph nodes are usually infected and involved cells have been found in the liver, bone marrow, spleen, kidney adrenals, pancrease and occasionally, cells in the heart and lung. Infarct lesions are often present in the lymph nodes and spleen which are thought to be caused by vascular occlusion by the swollen endothelium. The liver is reported to show necrosis around the central veins presumably also due to vascular occlusion (107). Erythrophagia is demonstrated by the cells of the reticulo-endothelial system and has been presented as evidence to explain the pathogenesis of the anemia.

If an intercurrent infection does not manifest itself, the patient's peripheral blood values stablize to normal and the patient appears to convalesce. However, the disease almost never terminates at this stage; Oroya Fever is usually followed after a variable period by Verruga Peruana.

B. Histoid Syndrome (Verruga Peruana).

Unlike Oroya Fever, Verruga Peruana is characterized by a distinctive appearance and diagnosis is readily established upon physical examination. The patient may have several or many nodular eruptions which vary in color from red to purple. They occur on both covered and exposed areas; the regions of predilection being the limbs and face.

The accompanying signs and symptoms are not conspicuous. There is a recurrence of anemia to a slight degree, but it is never severe. There is some pain due to the pressure from the nodules.

The verruga nodules vary considerably in appearance, shape, situation, size disposition and the general aspect of the eruption. The miliary type is small and projects above the dermis resembling a "rosy drop," or may be white, resembling hypertrophied papilla which gradually grows and becomes dark red and glossy (37). Histologically, the nodules of this type are either composed of histiocytic cells or are in a transition stage to angiocytic cells. The nodular type are deeply seated in the dermis and subcutaneous tissue. They are larger and at first do not project above the skin but during the course of its evolution, begins to resemble a furnucle (37). These are also in the histocytic or aniocytic stage. If the nodular type begins to erode, it is then known as the mulaire type. The nodules of this type are in the fibrocytic stage.

The histopatology of these nodules show progressive transition from pre-eruptive histiocytic stage, angiocytic stage. Histologically there are three findings which typify the verruga nodules: (a) numerous, newly formed capillaries, (b) proliferated endothelial cells and (c) the presence of B. bacilliformis in the tissue. The endothelial cells tend to form masses lying in edematous connective tissue with the entire area showing infiltration by lymphocytes, plasma cells and polymorphonuclear leukocytes. The organisms appear as bright red, bacillary to coccoid forms (Regand Fixed-Giemsa Stained) located within or around the endothelial cells.

C. Asymptomatic Bartonellosis

The third type of infection seen in human Bartonellosis, the asymptomatic form, is detectable only by blood culture and has not been discussed readily in the past. Weinman and Pinkerton (127) report the findings of such cases when investigating possible reservoirs of the disease. At Callahuanca, a small village in the endemic area, these workers obtained blood cultures and peripheral blood smears from a random group of fifty-three residents. These persons gave no sign nor symptom of the disease. However, positive blood cultures for B. bacilliformis were obtained from five of these individuals and interestingly, none of the blood films obtained from these five persons demonstrated the organism. Of these cases, three had no past history of the disease and the other two gave a history of Verruga Peruana six months previously. It was pointed out at this time that such individuals may constitute an important reservoir of the organism responsible for Bartonellosis.

III. Bartonella bacilliformis, the Etiological Agent of Bartonellosis

A. History.

In 1909, A. L. Barton (10) was the first to detect and describe the organism in the red blood cells of man. The name, *Bartonella bacilliformis*, was suggested by Strong et al in 1915 (108) and this is the commonly accepted designation. The Seventh Edition of *Bergey's Manual of Determinative Bacteriology* (17) places the organism in the Order *Rickettsiales*; Family *Bartonellaceae*; Genus *Bartonella* and the type species is *Bartonella bacilliformis*.

The organism is described as being pleomorphic, appearing as rod-shaped, coccoid, ring- or disc-shaped, filamentous and beaded microorganisms, usually less than three microns in the greatest dimension. They are Gram negative, non-acid-alcohol fast, and stain lightly or not at all with aniline dyes, but have been reported to stain with Giemsa's stain after methanol fixation. The organism is motile by means of unipolar lophotrichous flagella (80).

B. Cultural Characteristics.

Bartonella bacilliformis grows poorly, or not all, on ordinary media without the addition of blood, serum, plasma or other blood products. On blood-glucose-cystine agar (54) growth occurs in 4-5 days, either as minute, circular, clear, mucoid colonies, or as an opaque, finely granular mucoid film that has a tendency to outgrow the original boundaries of the inoculum. After prolonged incubation (43 days), similar growth is obtained on twenty per cent horse-blood agar slants. Supplemented semi-solid nutrient agar (36) yields grossly visible growth at 36-48 hours and very heavy growth at about the fifth day. Gieman's supplemented tryptone broth yeilds a granular turbidity with a finely divided, white, non-persistent sediment.

C. Physiological and Biochemical Characteristics.

All strains of B. bacilliformis studied by Noguchi et al (67) were reported to be obligate aerobes. The organism grows slowly in broth cultures without aeration or constant mechanical agitation.

The organism grows at 37° C and between 25-58° C. However, they continue to grow and survive longer at the lower temperatures (67).

The hydrogen ion concentration in which growth occurs lies between pH 6.8-8.4, with the best growth obtained at pH 7.8. Noguchi and Battistini (67) did not notice any morphological variation due to changes in the hydrogen ion concentration.

None of the common carbohydrates (glucose, sucrose, galactose, maltose, levulose, xylose, lactose, mannose, dulcitol, arabinose, raffinose, rhammose, dextrin, inulin, salicin, and amygdalin) are reported to be fermented (67,17).

D. Pathogenicity.

Bartonella bacilliformis has been employed in a number of studies concerned with its effects when inoculated into experimental, laboratory animals. These investigations were generally directed at attempts to reproduce the various forms of the disease (Oroya Fever, Verruga Peruana, and asymptomatic Bartonellosis) in animals, or to determine their relative susceptibilities or resistances to Bartonella bacilliformis. In the earlier studies, the inoculum usually consisted of: (a) the blood of an Oroya Fever patient, (b) tissue from a verrugous nodule, or (c) suspensions of the insect vector -- Phlebotomus verrucarum. Following the development of cultivation methods for Bartonella bacilliformis, the inoculum has quite often been cultures or suspensions of cultured organisms, and subsequently, the attempts to reproduce the disease have been somewhat more successful. Noguchi and Battistini state in an explanation of their more successful investigations: "It is possible that the overwhelming number of young, active organisms, such as are present in cultures, are necessary to break down the normal resistance, which is considerable. The relatively high resistance of such animals, as compared to man, may explain the fact......." (67).

The animals which have been used in pathogenicity studies are: (1) various species of monkeys (46,69,73,103,128,53,91,67,68), (2) donkeys (91,74), (3) dogs (113,74,102,122), (4) rabbits (128,122,91,107,67), (5) squirrels (42), (6) guinea pigs (122,91,67,102), (7) mice and rats (67,78) and (8) chickens (3,128).

In spite of frequent reports by natives of the endemic zone that certain domestic animals often present verruga nodules, such infections have not been reproduced experimentally (2). In addition, with one exception, no animal has been found naturally infected with B. bacilliformis. Hertig (46) cultivated this organism from the blood of a single field-mouse, Phyllotis sp., from the Rimac Valley in the endemic zone. Attempts to recover the organism from and to infect other individuals of the same and rodent species from the same area, have been unsuccessful.

The investigations of Noguchi are usually considered as the classical pathogenicity studies employing the Macacus rhesus monkey as the experimental animal. In 1926, Noguchi and Battistini were able to isolate B. bacilliformis from a patient with Oroya Fever and in turn challenged the Maracus rhesus with the live organism (67). One animal was inoculated I.V. and I.D. with the organism and three animals were subsequently inoculated with the organisms derived from a sequence of blood cultures from the first animal yielding positive growth of B. bacilliformis. The first animal showed an intermittent type of fever which was mild during the 55 days of observation and areas of induration at the site of I.D. injections. These were the only outward signs of remorse demonstrated by this animal. Blood cultures taken at 10 day intervals yielded positive cultures of B. bacilliformis and a small number of intracorpuscular forms of the organism were demonstrated in blood smears. One of the nodules which was excised 16 days post-inoculation, showed typical infiltration of mononuclear phagocytes but the organism was not observed. The two subsequently inoculated animals showed signs of a more severe type of intermittent fever, yielded positive blood cultures and demonstrated nodules at the site of I.D. inoculation which became exudative in one case. One animals died during the process of excising the I.D. nodule. Post morten findings were an enlarged spleen and lymph nodes but no lesions in the lungs were observed. Positive cultures for the organism were obtained from the autopsy material i.e., blood, spleen and lymph nodes, and the organism was demonstrated in small number "in the red blood corpuscles." Control inoculations made with the culture media and with killed cultures did not give any of the changes induced by the living organism. Filtration experiments with cultures were also reported negative.

Subsequent studies by Noguchi (70) employed the approach used in the above study. A saline suspension of a subcutaneous nodule (excised from a verruga patient) was refrigerated for 2 weeks and inoculated into 2 young rhesus monkeys, I.V. and I.D. Post-inoculation findings were irregular febrile reactions, enlargement of the lymph glands and in one instance a subcutaneous nodule arose, independently of direct inoculation on the tail. Bartonella bacilliformis was isolated from the blood of both animals and from the nodule. Three monkeys were subsequently inoculated with material from the first two animals, i.e., two with cultures of the organism isolated from blood and one with a saline suspension of the nodule. In the second passage the infection induced by local inoculation of cultures was reported as severe both locally and constitutionally and was accompanied by marked anemia. In the third animal, the "systemic infection was less severe but the local lesions were more striking."

In 1929, Noguchi, Mueller et al (74) inoculated a group of animals with crushed insects of the phlebotomi. The results were variable with the material inoculated but consistent with the material of "4 different lots of phelbotomi." The later infected animals yielded positive blood cultures for B. bacilliformis which produced typical verrucous lesions on subsequent inoculations into other monkeys.

The pathogenicity studies since this time have confirmed the investigations of Noguchi and his colleagues, in that in the inoculation of monkeys, epidermal lesions at the site of inoculation(s) and asymptomatic Bartonellosis (positive blood cultures with no anemic condition) are usually produced (53,128,74). Weinman and Pinkerton reported that they had produced the anemic form of Bartonellosis in one splenectomized monkey (128). This animal was inoculated intravenously with 2.0 ml whole blood from a patient with a fatal case of Oroya Fever. In addition, a small amount of the blood was injected into the inguinal lymph nodes. Death occurred 26 days after inoculation, preceded by a severe progressive anemia. At death, 7% of the red blood cells were parasitized with organisms resembling B. bacilliformis, each infected cell containing 1-12 organisms. On necropsy the animal was found to have pulmonary tuberculosis and hematophagous nematodes in the intestine, infections which in themselves could have accounted for the anemic condition. Noguchi (68) also reports of inducing an anemia of "extreme type" when employing organisms which had been passed through "susceptable animals." However, such a condition was reported to be secondary in nature.

The reported findings of the investigations utilizing other animals are far from uniform and are often contradictory. Epidermal lesions at the sites of intra-dermal inoculation have been produced directly in the donkey (74) and dog (74), with minced tissues of human verruga nodules and indirectly in the dog (74), colt (74) and squirrel (42), with the inoculation of cultures or suspensions of cultured organisms.

Guinea pigs, mice and rats are reported to be refractory to inoculation (67,78). Townsend (122) reported infections with B. bacilliformis in various animals, including guinea pigs and rabbits. However, the work of Townsend is generally not accepted as valid, the difficulty based on questionable histological findings (46). Rabbits are generally considered to be non-infectable, with the exception of local lesion development following intratesticular inoculation (107). Chickens are generally considered resistant to infection (3,127). However, in later studies utilizing developing chick embryos, lesions were consistently produced on the chorioallantoic membrane (55).

To recapitulate: monkeys, donkeys, dogs, rabbits, squirrels, guinea pigs, mice, rats and chickens have been utilized in previous pathogenicity studies. The mode of inoculation has varied. The inoculum has consisted of a) blood from severe Oroya Fever patients, b) material from verruga nodules, c) suspensions of trituarated insect vectors, and d) cultures or suspensions of cultured organisms. Verrugous lesions and asymptomatic Bartonellosis may be produced in almost all species of monkeys. With other animals, the results are not uniform and, in some instances, contradictory. Anemia has not been substantially produced in any laboratory animal.

"nota bene"

In the preceding section, the term "epidermal lesion" has been used frequently to describe the resulting cutaneous lesion obtained in laboratory animals when injecting (1) blood of an Oroya Fever patient, (2) tissue from a verrugous nodule, (3) suspensions of the insect vector or (4) cultures or suspensions of cultured organisms. However, when the various authors did produce a cutaneous lesion at the site of inoculation, the lesion was invariably called a verrugous nodule by them. In addition, some authors state that "the cutaneous form (Verruga Peruana) was produced" (128). Since cutaneous lesions at the site of intradermal inoculations can be obtained with many microorganisms or their metabolic products, it is misleading to use the term of the cutaneous form of Bartonellosis, Verruga Peruana, for the epidermal lesion produced in experimental animals. In addition, Verruga Peruana is considered to be a more general skin phenomena, nonlocalized, generally follows the anemic stage of the disease and is quite possibly the manifestation of a hypersensitive state. In no instance of the produced cutaneous lesions in laboratory animals are the above criteria substantiated.

To review, histologically there are three findings which typify the verruga nodules seen in human cases: (1) numerous newly formed capillaries, (2) proliferated endothelial cells and (3) the presence of Bartonella bacilliformis in tissue. The endothelial cells tend to form masses lying in edematous connective tissue, the whole area showing infiltration by lymphocytes, plasma cells and polymorphonuclear leukocytes. The organisms appear as bright red bacillary or coccoid forms (Regard fixed-Giemsa stained) located within or around the endothelial cells (130). Such a histological pattern has been observed in the experimental, epidermal lesions, but such a pattern does not totally substantiate labeling these lesions as the "cutaneous Verruga Peruana."

E. Epidemiology, Ecology, Modes of Transmission and Natural Reservoirs.

Bartonellosis is unique in its geographical distribution, being in all probability, confined not only to Peru, Columbia and Ecuador, but to certain restricted areas in these countries. The disease occurs between 8 and 13 degrees south latitude and an altitude varying from 2800 to 9000 feet, with the costal areas void of any indication of the disease. In addition, the disease appears only on the western slope of the Andes, in certain narrow ravines or canyons with luxuriant vegetation and considerable heat during the day. The small towns and villages where the disease is prevalent are sheltered from strong winds (which would affect insect life), as the ravines are at right angles to the prevailing winds. It is interesting to note that since the time when the name Bartonella bacilliformis was applied to the organism stated as the etiological agent of of Carrion"s Disease, the disease has persisted indefinitely in given areas. Earlier recognitions of the disease in these areas, thought to date back to 1860 and given more attention in 1870, further indicates the establishment of an infection and it being of only focal persistance, with little indication of spread.

The only extensive, reported epidemiological study was done by Gomez in 1914 (38). Bartonellosis is seen to occur in both sexes, all races and at all ages including the newborn, when it is usually of the severe type. Even though the disease is not considered hereditary, a case was seen which was contributed to an apparent intra-uterine infection. Gomez concluded that within the first ten years of life, the disease is of the benign type, reaching cases of severeity in adolescence and adult life. In the verrugous areas, practically all natives are infected during early childhood, this infection conferring to the person in adult life a lasting immunity (with rate exceptions). Most of the well-marked cases of the disease are seen in strangers to the areas who remain there during the night. While the disease may be acquired at any time of the year, the majority of infections occur between December and March.

It is the consensus in Peru that many animals suffer from the eruptive type of Carrion's Disease, but relatively few investigations have been made regarding animals as a natural reservoir of the organism. Even though various animals can be artificially infected, such evidence is no proof the animals can acquire the disease naturally. Even though the disease can be transferred through several generations of animals (53), further experimental evidence is needed to show whether all the developmental stages of the organisms are communicable and whether a particular state of the infectable host(s) is required.

Since Bartonellosis is restricted to protected, mountainous areas, an insect vector has been implicated in the transmission of the disease. The first suggestion that Carrion's Disease might be transmitted by some blood sucking insect was made by Arce in 1889 (34), and the first extensive investigations were made by Townsend (124) in 1915. He demonstrated that the disease was transmissible by a species of phlebotomus, which he named _P. verrucarum_, and was able to exclude mosquitoes, flies, lice, bedbugs, ticks and mites as possible vectors. In agreement with Townsend's views, Shannon (103) gave ecological evidence that phlebotomus is the vector of Verruga Peruana. Since the disease is contracted only in certain areas, predominately during the night, both indoors and far from habitation, at any time of the year, Shannon concluded that the vectors must be common blood suckers of man, restricted to given areas, nocturnal in habits and able to breed in unrestricted situations. He also added that since the adult has a restricted flight, they must continue active throughout the year to conform to the above mentioned criteria.

At this time, Shannon added another species of phlebotomus regarding the transmission of _Bartonella bacilliformis_, _P. Noguchii_. However, if the phlebotomi are proven vectors of this disease, there are other species serving as vectors since the disease occurs in the areas where _P. verrucarum_ is absent but where other species of phlebotomus are found. In 1929, Noguchi and co-workers (73) inoculated a group of animals with crushed insects of the phlebotomi. The results were variable with the material inoculated but consistent with the material of "4" different "lots" of phlebotomi. The latter infected animals yielded positive blood cultures for _B. bacilliformis_ which produced typical verrucous lesions on subsequent inoculations into monkeys. From such evidence, it is clear that much further investigation is required to clarify the status of the phlebotomus in Bartonellosis. Numerous investigations have been concerned with the phlebotomi as possible vectors of B. bacilliformis (11,44,45,46, 47,48,60,99,100,116,117,118,119,120,121,122,132,133). However, many facets concerning their definite role in transmission and the ecology of the disease remain undecided. Since there are relatively few animals and a sparse human population in most areas where the disease is endemic, possible, natural reservoirs have been implicated as a source of the organism (40,124). Maldonado (59) found that certain plants were characteristic of the endemic zone and that they did not exist in non-verrugous areas. Such findings suggest that these plants may act as a reservoir of the organism or as a source of food for the insect vector. Mackehenie and Coronado (57) found in the latex of certain plants and in the digestive tracts of small insects which feed on these plants, organisms resembling B. bacilliformis. These organisms were cultivated and gave an agglutination with serum from several verruga patients (convalescent). However, Koch's postulates were not fulfilled.

F. Serology.

The first serological studies in direct concern with Bartonellosis are those of Biffi and Gastiaburu in 1903 (13). This and subsequent studies (35,65) were undertaken to investigate the existance of auto-agglutinins and auto-hemolysins as possible causes for the massive destruction of red blood cells in human Bartonellosis. Employing various methods known at this early time, these workers were unable to demonstrate such agglutinins and hemolysins in the disease.

Guzman-Barron (39) reported that he found auto-agglutinins to be present in low titer in those instances in which the anemic stage was followed by the eruptive period. When no preceding anemia was present, auto-agglutinins were not demonstrable. Auto-hemolysins could not be demonstrated during either of the two clinical types of infection nor when the eruptive stage was preceded by the anemic form of the disease.

Although additional studies were made of the hemolytic anemia, characteristic of Bartonellosis, some aspects of the hemolytic processes could not be adequately studied until recent developments in hemotologic and serologic techniques became available. Studies of Reynafarje and Romos (88,89,90) exclude the possibility that an immunologic mechanism causes the anemia. Such information is based on extensive studies involving immunohemotologic techniques. To investigate the existence of auto-agglutinins in human Bartonellosis, both the direct and the indirect Coomb's test were applied in instances of the anemia and eruptive forms of the disease. In addition, tests were made for cold agglutinins and hemolysins. All tests were reported negative.

Subsequent serological studies of Bartonella bacilliformis have been oriented towards the organism itself and its antigens rather than to the hemolytic disorder, i.e., that the anemia may be explained on the basis of an immunologic mechanism.

Strong, et al (108) prepared an antigen from an isolate of _B. bacilliformis_ taken from a verruga nodule. When this antigen was used in the complement-fixative test (C-F), these workers were unable to detect C-F antibodies in the sera of nine patients with the eruptive form of Bartonellosis. In direct contrast to these findings, Aldana (3) using a cultural antigen of _B. bacilliformis_, obtained positive complement fixation with convalescent sera of patients previously demonstrating the eruptive form of the disease.

Owing to the fact that the masses of organisms, typifying the _in vitro_ growth of _Bartonella bacilliformis_, are difficult to disperse, suitable suspensions of the organism for the agglutination test are difficult to obtain. Therefore, the majority of serologic tests have employed the complement fixative test. In 1928, Noguchi obtained immune rabbit sera by inoculating the animals at intervals with a live culture suspension (72). In turn, the immune sera were reacted with suspensions of various strains of _B. bacilliformis_, which had been heated at 60° C to assure the use of killed organisms. All of the strains demonstrated complete fixation with the antiserum with the exception of one strain, which gave partial fixation. These results were interpreted by Noguchi to indicate "that on the whole, the strains belong to the same serologic group."

A more recent investigation by Reese and co-workers (87) employing the complement fixation test was undertaken to investigate possible relationships between _B. bacilliformis_ and the _Rickettsiae_. Satisfactory C-F antigens were prepared by heat treatment of aqueous suspensions of the organism. It was found that various strains of this organism, cultivated _in vitro_ for long periods were as capable of producing C-F antibodies in the rabbit as were recently isolated strains. Five strains of _B. bacilliformis_ could not be differentiated on the basis of quantitative complement fixation, leading the authors to agree with Noguchi in that there is considerable degree of antigenic similarity in strains of _B. bacilliformis_. These workers were unable to detect agglutinogens for Proteus OX19, OX2 and OXK, utilizing the Weil-Felix reaction.

As previously mentioned, suitable suspensions for the agglutination test are difficult to obtain. However, some attempts have been made to demonstrate an agglutinogen(s) of _B. bacilliformis_, employing techniques to obtain a uniform suspension of the organism with a regular distribution of single organisms. Howe (52) found that if the organism is suspended in physiological, unbuffered saline, it can be stored as a coarse suspension which can easily be dispersed with a pipette. The coarser particles were allowed to settle out and the resulting suspension consisted of individual cells with occasional clumps consisting of 5-10 cells, as revealed by dark field microscopy. The conventional agglutination test was employed and the titer determined through the use of dark field examination. Specific agglutinins were obtained by injecting a series of rabbits with living cultures of _B. bacilliformis_. The organisms were those cultivated for 7-14 days. Each animal received 12-13 intravenous inoculations over a 60 day period. At no time did any of the animals show signs of systemic reaction which could have been interpreted as having resulted from the inoculations.

The inoculated animals were found to produce a high titer of specific agglutinins which, under the conditions of the experiment, began to decline after about one month following the last inoculation. In addition, sera from six patients, taken in the different stages of Bartonellosis were shown to contain a low but definite titer of circulating antibody by means of the agglutination test.

In 1943, Howe and Hertig, reported on limited experiments aimed at ascertaining the degree to which active immunization may protect against infection with B. bacilliformis (51). A group of 22 military personel, without previous history of Bartonellosis, and presumable non-immune, were inoculated subcutaneously with 1 ml of a formalized vaccine prior to being stationed in the "verruga zone." The majority of these guards received 1-2 more injections of vaccine during the first two weeks of their duty. In all instances, the inoculations resulted in the appearance of a high titer of homologous circulating agglutinins. Twelve of these men on at least one occasion during the period of observation, demonstrated positive blood cultures for B. bacilliformis. Five out of the twelve developed a mild degree of the skin eruptions, all had periods of mild systemic symptoms and one required hospitalization.

Comparison of these studies with observations made on unvaccinated personel were interpreted by the authors as warranting the belief that active immunization may definitely lessen the course of a potentially serious disease, although not preventing infection. The protective effect of the vaccine was considered not to be directly related to the specific serum agglutinins which resulted from administration. In addition, the agglutinins produced by the vaccine and those produced by natural infection were indistinguishable. Thus, the type of antigen, if any, which causes the production of protective antibody, remains unclear. From an extensive study of the immunological response to natural infection (49,50), agglutinins were insignificant in acquired immunity, per se, although they did occur in most cases during the acute stages of Bartonellosis, in the anemic or eruptive phases. However, no clear-cut relationship is seen between the agglutinin titer and the severity of the disease. This is seen when only 9 out of 203 inhabitants of endemic regions (4.4%) were found to harbor the organism in their blood without symptoms or sign of Bartonellosis and one of these cases showed specific agglutinins.

G. Taxonomy.

Following the early description (10) and ultimate naming (107) of the organism now known as Bartonella bacilliformis, there has been some discussion concerning the inclusion of the organism with a number of animal parasites, in one family, Bartonellaceae. The Bartonellaceae consists of a group of blood parasites characterized by their smallness and their position adjacent to the red blood cell. Bartonella bacilliformis, an organism accepted as being found in only restricted areas of Peru, Colombia and Equador, may be cultured on artificial media and was early recognized by Noguchi (70) as a true bacterium. In direct contrast, the other members of the family Bartonellaceae may be found universally as animal parasites which cause a latent infection activated by splenectomy, cannot be cultured with certainty on artificial media and are still without a satisfactory taxonomic position. Weinman (129), although listing all Bartonellaceae as bacteria, contrasts the biologic traits of B. bacilliformis with those of the animal Bartonella (Hemobartonella). In this article with Bengtson in the Sixth Edition of Bergey's Manual (16), he listed three families with the order of Rickettsiales: Rickettsiaceae, Bartonellaceae and Chlamydozoaceae. The family Bartonellaceae is comprised of four genera: Bartonella, with the single species B. bacilliformis; Haemobartonella (animal Bartonella); Eperythrozoan; and Grahamella. Even though the biologic characterizations described above contrasts these organisms, the Seventh Edition of Bergey's Manual (17) continued the classification used in the Sixth Edition.

In discussing the experimental evidence characterizing B. bacilliformis, Haemobartonella muris and Eperythrozoan coccoides, Peters and Wigand (79) advised setting the animal Bartonellaceae farther apart from Bartonella bacilliformis. In addition, they suggested retaining B. bacilliformis among the true bacteria but excluding H. muris and E. coccoides. B. bacilliformis has characteristics typical of true bacteria, including size and morphology, growth on culture media, propogation by binary fission, cell walls, and behavior in serological tests. There are no just criteria to include H. muris and E. coccoides with the true bacteria. In addition, they can be set apart from the protozoa on account of their small size and lack of cellular structure, and differ from the pleuropneumonia-like organisms in that the latter can be cultured on artificial media.

A relationship to the Rickettsiae, often supposed because of analogies in size and transmission by insects should be excluded. The Rickettsiae definitely differ from B. bacilliformis, which is flagellated and can be cultivated on artificial media, and also, in structural details from H. muris and E. coccoides. Multiplication of the Bartonellaceae in insect vectors is questionable and moreover, transmission per se by insects is too common in protozoa, bacteria and viruses to form a criterion for taxonomy.

H. Fine Structure.

Since light microscopy is inadequate for studying the minute structural details of B. bacilliformis, ranging between the size of Rickettsiae and that of the large viruses, studies employing the techniques of electron microspy have been utilized (80). Through such studies, the contrast between B bacilliformis and the species H. muris and E. coccoides is striking. B. bacilliformis (from culture) shows retracted cytoplasm and cell walls typical of true bacteria. Also like bacteria, they are rod-shaped in young cultures and mostly coccoid in older ones. In comparison studies with animal Bartonella (Haemobartonella), B. bacilliformis was studied as a blood parasite. As such, it was primarily rod-shaped with cell walls visible. The annular and coccoid particles seen also in light microscopy (129) are considered to be degenerate forms. Such structures preferably occur during the final stages of infection, with B. bacilliformis, under antibiotic treatment and in old cultures.

The action of crystallized enzymes on fixed organisms has also been studied (80). Following trypsin treatment, B. bacilliformis shows distinct cell walls, indicated from the absence of plasmatic substances, which are also seen in other Gram-negative bacteria (81,82).

The motility of B. bacilliformis observed in blood (108) and in cultures (57,80) was found to be due to unipolar flagella. However, the presence of flagella were not demonstrable in blood material. The diameter and arrangement of the flagella resemble those found in motile, true bacteria.

I. Treatment.

The anemia induced by Bartonella bacilliformis has a very severe prognosis due largely to the occurrence of intercurrent infection by various organisms (94,96,97). It has been pointed out that there is little or no experimental evidence to indicate a specific antibiotic which acts against B. bacilliformis (28,96) and therefore, the treatment used is symptomatic. Payne and Urteoga (77) suggest that chloramphenicol may be effective in Bartonellosis alone, without evidence of a secondary infection.

Since the majority of patients are seen in the latter stages of anemia, it is difficult to contribute recovery to immunity,spontaneity or antibiotic therapy. However, as pointed out by Cuadra (28) and contrary to a high fatality rate in complicated bartonellosis, uncomplicated Bartonellosis has an excellent prognosis. Therefore, the main objectives in the treatment of Bartonellosis should be the prevention and control of intercurrent infections. Penicillin (63,30,4), Streptomycin (5,30,66), and the Tetracycline (137), have been used for such measures but Aldana (6) points out that complications of Salmonella infection often cause a fatal termination following such therapy. Thus, Cuadra (30) states that, "at present chloramphenicol is the most suitable antibiotic available for the treatment of acute Bartonellosis because it is the only effective drug against the intercurrent Salmonella infection."

MATERIALS AND METHODS

I. Cultures and Sources

The following strains of Bartonella bacilliformis were used in these studies:

ORIGINAL STRAIN NUMBER	YEAR ISOLATED[1]	COMPLETE CODE NUMBER
267[2]	1939	800
372[2]	1939	13
JU	1957	200-44t[3]
J27	1957	11-40t
020	1957	400-40t
VS-320	1964	500-6t
C-1	1964	600-5t
C-2	1964	700-5t
VS-15	1950	14
151	1959	17-40t
172	1959	10-40t
157	1959	300-44t
214	1959	900-44t
208	Unknown	15
529	Unknown	16
L-1	1964	12-3t
Unknown[5]	Unknown	100
020	1957	KC-583
157	1959	KC-584

[1]Unless otherwise stated, all isolates were obtained through the courtesy of Dr. A. Herrer (National Institue of Hygiene and Public Health, Lima, Peru and Dr. M. Cuadra (Hospital Dos de Mayo, Lima, Peru).

[2]Isolated from sandflies.

[3]Number preceding "t" indicates known number of times isolate had been transferred prior to the time received in our laboratories.

[4]Isolated by Dr. J. Slack and P. Mitchell, West Virginia University Medical Center, Morgantown, West Virginia.

[5]Obtained from Dr. D. Weinman, Yale University.

[6]Obtained from Miss Elizabeth O. King, Communicable Disease Center, Chamblee, Georgia (original numbers indicate same isolates of Dr. A. Herrer, Lima, Peru).

II. Identification Procedures

A. Staining Techniques

The following standard procedures of staining methods as outlined in the *Manual of Methods for Pure Culture Study of Bacteria* were used with the exception of the Giemsa staining method (26).

1. Benian's Congo Red Negative Stain
2. Gram's Stain
3. Ziehl-Neelsen's Acid-Fast Stain
4. Macchiavello's Stain for Rickettsiae
5. Methylene Blue Stain

The Giemsa staining method used is as outlined below.

Buffer solutions (combined)[1]............ 100 ml
Stock Giemsa solution.................... 5 ml

[1] Na_2HPO_4, 11.88 gm/liter distilled water
KH_2PO_4, 9.08 gm/liter distilled water

Combined prior to use in such a ratio as to give a final pH of 7.3.

Smears of organisms from culture or peripheral blood smears are allowed to air-dry. Slides are then fixed in absolute methanol for ten minutes, allowed to air-dry and then stained with the above solution for one hour. The slides are washed with tap water, followed by a distilled water wash and examined microscopically.

B. Microscopy

Standard techniques of dark field and phase microscopy were used to study the morphological characteristics of *B. bacilliformis*. Cultures of the various isolates were examined at regular intervals in various media to determine the effects of time and nutrients on the morphology of the organism respectively. Dark field microscopy was also used to examine the peripheral blood of experimentally infected animals for the presence of the organism. In such instances, EDTA collected blood was diluted in physiological saline in order to obtain a preparation conducive to dark field illumination.

C. Fluroescent Antibody Techniques (FA)

Antisera of hyper-immunized rabbits which had been injected with Strain

500 were pooled. The globulins were fractionated and conjugated with fluorescein isothiocyanate in accord with the method of Cherry et al (25). Protein content of the precipitate was determined by the standard Biuret method on a B and L "Spectronic 20" spectrophotometer; a ratio of 1 to 40 dye to protein was used in conjugation and unconjugated fluorescein was removed by passing through a Sephadex G25 column. Normal serum globulin was conjugated in the same manner.

1. FA technique in staining of cultivated organisms

Organisms suspended in physiological saline were placed on a slide and allowed to air-dry. They were then slightly heat fixed and stained for 15 minutes with undiluted conjugated serum, after which excess dye was removed through two phosphate buffered saline (pH 7.4) washes. They were then rinsed in triple distilled water to remove the saline, mounted using glycerine in the same phosphate buffer and examined under ultraviolet light (Leitz Ortholux Microscope, HBO 200 light source, 2 mm UGI exciter filter and Euphos Garner barrier filter, E. Leitz, Inc., New York). Smears for the fluorescence inhibition test were allowed to react for 12 minutes with unconjugated, immune sera prior to staining.

2. FA technique on frozen tissue sections

Tissues of experimentally infected animals were removed en-bloc and sections immediately imbedded and frozen in gelatin. Six to eight micron sections were cut on a Cryostat freezing microtome and placed on alcohol cleaned slides containing a thin film of egg albumin. The sections were then stained by the direct FA method and examined under ultraviolet light.

D. Hemagglutination

The hemagglutination procedure employed was basically that of Salk (101). Organisms cultivated in the to-be-described over-lay medium were collected via centrifugation, resuspended and washed three times in sterile, physiological saline (pH 7.0). The resultant packed cells from the third washing were then resuspended in sterile saline to give an approximate concentration of 10^9 cells per ml, according to the McFarland Scale. Doubling dilutions of this suspension were made in Phosphate Buffered Saline (PBS), pH 7.0, in 0.5 ml amounts. 0.5 ml amounts of a 0.35 per cent suspension of washed human O positive red blood cells were then added to each dilution. Routine controls were included in the test system with readings made after one hour incubation at R°. The results were read according to the "pattern" produced and were recorded as plus (+) meaning complete, plus-minus (±) referring to partial and minus (-) indicating no hemagglutination. The end-point was taken as the highest dilution of the organism producing maximal hemagglutination and the titer expressed as the reciprocal of the highest dilution.

E. Agglutination

Isolates of B. bacilliformis were more precisely identified and antibodies more accurately titrated by the conventional tube agglutination technique than with the microscopic slide agglutination test. In addition, since the organism has a tendency to clump, the tube agglutination test could be interpreted more accurately.

The procedure used was a standard technique employing constant, given amounts of antigen, usually a viable, intact cell suspension, and varying dilutions of anti-serum. Anti-sera of experimentally infected animals were routinely processed by this method.

F. Immuno-Diffusion

The immuno-diffusion method used was a modification of the Ouchterlony technique (76). Ion Agar No. 2 (Oxoid) was dissolved in distilled water by boiling to give a final concentration of 1.0 per cent. Clean, 2 x 2 inch projector slide cover glasses were immersed in the hot agar solution for five seconds and dried at R° for one hour. The agar employed in the test medium was 0.8 per cent Ion Agar No. 2 prepared as follows:

Distilled water..........1 liter
Ion Agar No. 2...........8.0 gm
Sodium Chloride..........8.5 gm
Glycine.................75.0 gm

The mixture was then heated to boiling for ten minutes and kept at 80° C in a water bath while preparing the plates. According to the pattern used in the various studies, 8 mm assay cylinders were arranged on the coated plates so that a distance of 8 mm separated the wells to be used to demonstrate antigen-antibody systems. Eight ml of agar was then added to each plate and allowed to solidify at R°. After approximately two hours, the assay cylinders were carefully removed from the plate to give resulting, uniform wells. Following the addition of the reactants, all plates were incubated at 4° C.

III. Cultural Techniques

A. Agar Slant Tissue Culture

One particular medium employed in the cultivation of B. bacilliformis is an agar slant tissue culture medium employing minced mouse embryo. Such a technique has been described earlier by Zinsser (138). A double strength

Tyrode solution[1] was prepared except that only 1.0 gram of sodium bicarbonate was used per liter. A mixture of 75 ml of said Tyrode solution and 55 ml rabbit serum to which 4 ml of a phenol red solution has been added was filtered through a Seitz filter. To the filtrate was then added 80 ml of the sterilized Proteose Peptone Nutrient Agar[2] cooled to 45+ C and the pH adjusted to 7.4-7.6 with 10 per cent sodium bicarbonate or 0.3 N hydrochloric acid. The medium was dispensed in 4-6 ml amounts into glass tubes fitted with rubber lines screw-caps and slanted to give a long surface. The medium was stored at 4° C until used.

To prepare the inoculum, 8-10 day mouse embryos were decapitated and the body portions minced to give a resultant tissue matrix. An ordinary 2 mm bacteriological loop was used to transfer the tissue matrix to the surface of the prepared agar slant. Two to three loopfuls per slant were sufficient. The resulting tissue "heaps" were then inoculated with viable, cultured cells of _B. bacilliformis_, incubated in a slanted position at 28° C and observed daily for bacterial growth by staining small portions of the tissue matrix with the Giemsa stain.

B. Semi-Solid Medium

A medium which supports excellent growth of _B. bacilliformis_ and is useful in maintaining stock cultures of the organism is a semi-solid medium supplemented with blood products. Such a medium was used earlier by Noguchi and Battistini (67) in the cultivation of B. bacilliformis. The medium is prepared as follows:

Sterile Physiological Saline (pH 7.8)	660 ml
Sterile Rabbit Serum	60 ml
Nutrient Agar Solution[3]	80 ml
Hemoglobin Solution[4]	75 ml

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[1]
NaCl	16.0 gm
KCl	0.4 gm
$CaCl_2$	0.4 gm
$MgCl_2$	0.2 gm
NaH_2PO_4	0.1 gm
$NaHCO_3$	1.0 gm
Glucose	1.0 gm
Distilled H_2O	1 liter

[2]
Proteose Peptone No. 3 (Difco)	4.8 gm
Glucose	0.12 gm
Sodium Chloride	18.75 gm
Bacto Agar (Difco)	22.50 gm
Distilled Water	2 liters

[3]
Proteose Peptone No. 3 (Difco)	12.0 gm
Bacto Agar (Difco)	13.5 gm
Glucose	0.3 gm
Sodium Chloride	3.0 gm
Distilled Water	600 ml

Autoclaved sterilization

[4]Three parts sterile distilled water to one part fresh, sterile defibrinated rabbit blood

Sterile, melted nutrient agar was added to the sterile saline which had been heated to 56° C. The mixture was allowed to cool to 45° C at which time the sterile serum and hemoglobin solution were added. The medium was then dispensed into sterile, cotton plugged tubes (13 x 100 mm), 5-6 ml per tube and sterility checked at R° for 24-48 hours. Tubes of the sterile medium were then inoculated with viable cells of B. bacilliformis, shaken to insure a disperse inoculum, rubber-stoppered and incubated at 28° C. Growth was observed grossly; occurring as distinct micro-aerophilic, white-gray bands.

This particular medium was also used in procedures of isolating B. bacilliformis from experimentally infected animals. One to two ml of whole blood was obtained from such animals aseptically, allowed to clot and portions of the clot were then transferred to the medium. Growth usually occurred within seven to ten days.

C. Solid Medium

A solid medium used in the cultivation of B. bacilliformis was prepared as follows:

Heart Infusion Broth (Difco)...	2.5 gm
Purified Agar (Difco)..........	1.5 gm
Glycerol.......................	1.0 ml
Distilled Water................	100 ml

The pH of the medium was adjusted to 7.6-7.8 and autoclaved for 18-20 minutes at 10 pounds pressure. Following sterilization, the medium was allowed to cool to 45° C and supplemented with fresh, sterile defibrinated rabbit blood to give a final concentration of 18-20 per cent. Plates were poured and conventionally inoculated with the streak technique and incubated at 28° C.

D. Over-Lay Medium

There was developed in our laboratories a liquid medium which supports excellent growth of B. bacilliformis. The medium consists of a layer of agar which is over-layed with a layer of broth medium and is referred to as O-L Medium.

The agar was prepared as follows:

Proteose Pepton No. 3 (Difco).....	4.80 gm
Glucose...........................	0.12 gm
Sodium Chloride...................	18.00 gm
Bacto Agar (Difco)................	30.00 gm
Distilled Water...................	2 liters

The medium was dispensed into 1-liter, screw-capped Erlenmeyer flasks to give a depth of 15-20 mm, autoclaved for 18 minutes at 10 pounds pressure and allowed to solidify. The broth was prepared in the same manner as the

Proteose Peptone agar with the exception of the Bacto Agar being eliminated. The medium was sterilized and following autoclaving, was allowed to cool to 50° C. It was then supplemented with 8-10 per cent sterile rabbit serum and 4 per cent sterile hemoglobin solution prepared from defibrinated rabbit blood, one part, to three parts sterile distilled water (pH 7.0). The supplemented liquid medium was then aseptically over-layed onto the solid medium to a depth of 20-25 mm. Organisms cultivated in the previously described media were inoculated directly into the O-L Medium and the flasks incubated at 28° C.

E. Embryonating Avian Eggs

Previous reports employing the use of embryonated eggs in culturing B. bacilliformis are few in number and not of an experimental nature per se (55). Since the factors influencing the growth of organisms in the embryonated egg are numerous, it was also the purpose of this study to determine the optimal conditions for cultivation of B. bacilliformis in the egg. The conditions which were varied are as follows: (1) age of embryo, (2) route of inoculation, (3) temperature of incubation and (4) source of inocula. Standard techniques as outlined in Experimental Virology (62) were employed in inoculation procedures and collection techniques.

F. Tissue Cultures

1. Culture media and reagents

Trypsinized Primary Monkey Kidney (PMK) cell suspensions were obtained from Microbiological Associates, Bethesda, Maryland and grown in Eagle's medium without the addition of antibiotics. The following componenets added aseptically in a sterile 250 ml graduated cylinder:

Eagle's Salt Solution (10x)..........	100 ml
$NaHCO_3$ (2.8 per cent)................	40 ml
Calf Serum (Microbiological Asso.)...	20 ml
BME Vitamin Stock (100x).............	10 ml
BME Amino Acids (100x)...............	10 ml
Glutamine (100x).....................	10 ml

Maintenance medium was prepared in the same manner as the above except calf serum was used in a 0.5 per cent concentration and arginine was substituted for glutamine in the same concentration. Eagle's salt solution was prepared in the following manner:

Solution I.

NaCl............	68.40 gm
KCl.............	3.72 gm
$NaH_2PO_4 \cdot H_2O$......	1.38 gm
Glucose..........	10.00 gm
Phenol Red.......	0.20 gm
Triple Distilled Water (TDW).	700 ml

Solution II.

$CaCl_2 \cdot 2H_2O$....... 1.50 gm
TDW............. 300 ml

The two solutions were autoclaved separately for 15 minutes at 15 pounds pressure, combined asceptically and refrigerated in 100 ml aliquots.

Sodium bicarbonate (2.8 per cent) was prepared in the following manner.

NaHCO ....56.0 gm
1 per cent phenol red..... 4.0 gm
TDW...................... 2 liters

After mixing, the solution was filtered through a Selas micro-porous porcelain bacteriological filter (Porosity #02) into a sterile flask, dispensed in 100 ml aliquots and refrigerated.

Glutamine was prepared by adding 14.6 gm of L-Glutamine to 500 ml TDW. After the glutamine was completely dissolved, the solution was filtered as above in a Selas bacteriological filter.

Trypsin used in cell culture work was prepared as follows:

1. Twenty-five gms of trypsin (Difco 1:250) was dissolved into a paste with a small amount of GKN[1] salt solution without Mg.

2. The paste was then dissolved in 900 ml warm GKN and 30 ml of 2.8 per cent $NaHCO_3$ was then added.

3. The final volume was brought up to a liter with GKN, filtered through a Selas (#02) filter, dispensed in 100 ml amounts and frozen. The resultant solution is 10x stock and is diluted 1:10 for trypsinization work.

[1]NaCl...........80.0 gm
KCl............ 4.0 gm
Glucose........10.0 gm
TDW............ 1 liter

The solution is sterilized by autoclaving and refrigerated until use.

2. Cell cultures

Primary Monkey Kidney (PMK) cells were received in 50 ml quantities containing 1 x 10^6 cells per ml. Primary bottle cultures were prepared immediately from these by centrifuging the suspension (1000 rpm for 10 minutes), resuspending the cells with 200 ml Eagle's growth medium and introducing 10 ml of the suspension into each of 20 sterile four ounce bottles. The bottles were then placed flat side down in a 37° C incubator.

Roller tube cultures were prepared from the primary bottle cultures when confluent monolayers of cells were observed (5-7 days). When such cell growth was noted, the bottles were removed from the incubator, the growth medium discarded and 5 ml of 0.25 per cent trypsin was added to each bottle. The bottles were gently agitated and observed until the cell sheet floated free of the glass. The clumps of cells were then dispersed through repeated pipettings, and finally transferred to sterile centrifuge tubes and collected at 1000 rpm for 10 minutes. The trypsin was removed by suction and the cell button resuspended in 5-6 ml growth medium. The resuspended cells were added to a sufficient amount of growth medium in order to plant 25 roller tubes per bottle, using 1.5 ml per tube. Roller tubes were stoppered with white latex, non-toxic stoppers (West and Co., Phoenixville, Pa.). Tubes were placed in roller drum at 37° C and held stationary overnight to allow the cells to adhere to the glass. The drum was turned on 8-12 hours later.

HeLa and HEP 2 continuous cell cultures are maintained in this laboratory and the procedure for such cultures is essentially the same as for planting of the PMK cells. The only notable exception is that 30-50 tubes were plated per bottle culture.

Approximately 2-3 days after planting, the growth medium was removed and replaced with maintenance medium. Dilutions of B. bacilliformis were prepared in the maintenance medium and 0.5 ml of each dilution were inoculated into each of 6 tubes of the tissue culture under question. Tubes were then incubated at 30° and 37° C., examined daily for evidence of cytopathogenic effect (CPE) and held for a total of 15-20 days. Maintenance medium and cells were routinely examined by Giemsa's staining procedure for the detection of the organism.

The procedure for the preparation of the MET's was essentially the same as the procedure outlined in Experimental Virology (62) for chick embryo tissue cultures. Eight to ten day old embryos were employed routinely. Comp te cell monolayers were inoculated and examined as described above ne presence of B. bacilliformis.

IV. Animals

Various species of laboratory animals were employed during the course of these investigation. They are listed as follows:

1. Male, Albino rabbits weighing 6-7 pounds unless specified differently in particular studies.

2. Rhesus Monkey, sex undetermined and weighing approximately 5-8 pounds.

3. Male, Albino guinea pigs weighing 500-600 grams.

4. Suckling mice, 2-5 days old.

5. Golden Hampsters weighing 200-300 grams.

Due to the various studies, specific modes of inoculation will be presented in discussion form when introducing the results of a given study.

V. Preparation of Inocula

A. Viable Cell Suspensions for Pathogenicity Studies

During the course of these investigations, numerous strains of *B. bacilliformis* were employed and were cultivated and prepared in various manners. According to the particular studies presented in the *Results*, such preparative information is as outlined below.

1. Pathogenicity Study I

To obtain an adequate inoculum for these studies, Strain 100 was grown for 72 hours in the following medium:

Tryptone Broth (Difco)......	1.5 gm
Fresh rabbit serum..........	50.0 ml
Distilled water.............	150.0 ml
pH 7.8	

In order to exclude the possibility of inclusion in the inoculum of any antigenic and/or irritating substance(s) which might be present in the medium, the cells were washed three times with phosphate buffered saline. A quantity of washed, resuspended packed cells sufficient to obtain an adequate cell suspension, comparable to a Number 4 McFarland Standard, was prepared.

The inoculations consisted of massive injections in an attempt to (1) overcome the natural high resistance of the rabbit and thereby produce effects which might otherwise not be manifested and (2) produce immune sera. Complete Freund's adjuvant was included in the first injection in hopes of producing a good local response and of providing a sustained stimulus for antibody production. The following was the injection schedule:

1st day - 1 ml cell suspension plus 1 ml adjuvant I.M.
2nd day - 1 ml antigen I.V. (marginal ear vein)
3rd day - 2 ml antigen I.V.
4th day - 4 ml antigen I.V.
5th day - 8 ml antigen I.V.
6th day - 8 ml antigen I.V.

2. Pathogenicity Study II

Cell suspensions of Strain 100 were prepared and injected in the same manner as previously described except that the washed, packed cells were resuspended in Tween-80 Tris buffered saline prior to use. Due to the natural clumping tendency of this organism and also the tendency of the organism to adhere to the glass walls of the container, some difficulty was encountered in obtaining a smooth cell suspension desirable for injection purposes. Such tendencies were eliminated with the use of the Tween-80 Tris buffered saline as the suspending medium.

Cell suspensions containing approximately 1 x 10^9 cells per ml were in addition standardized according to their hemagglutination titer and corresponding optical density (O.D.). The suspension titered at 1:16 with an O.D. of 0.602. Prior to inoculations, all animals employed in this study were subjected to a series of base-line studies so as to give comparative hematologic data.

3. Pathogenicity Study III

This study employed the use of organisms of the same strain, prepared in the same manner according to the methods given previously. The agents employed in conjuction with the injections were Corticotropin (ACTH), pig gastric mucin and 6-mercaptly purine (MCP). These agents were mixed with the organisms just prior to inoculation and were used in the amounts given in the following list of daily, I.V. inoculation schedules:

Rabbits 201 and 202 (without agents)

1 ml suspension of organism
2 ml
4 ml
8 ml
8 ml

Rabbit 203

2 ml suspension of organism plus	20 units ACTH
4 ml	20
4 ml	10
6 ml	10
8 ml	15

Rabbit 205 (Mucin)

1 ml suspension of organism plus	2 ml 5 per cent mucin
2 ml	4 ml
4 ml	4 ml
6 ml	2 ml
8 ml suspension of organism only	

Rabbit 206 (MCP)

1 ml suspension of organism plus 10 mg MCP
2 ml
4 ml
8 ml
8 ml

4. Pathogenicity Study IV

This series of pathogenicity studies employed the Rhesus monkey as test animal. Cell suspensions for purposes of inoculation of Strain KC-584 were prepared as follows:

Heart infusion agar plates described earlier were inoculated and incubated at 25° C under 5 per cent CO . After 7-10 days, the growth was removed from the agar plates with sterile saline and washed via centrifugation. The resulting packed cells were resuspended in sterile saline (pH 7.2).

The animals under question were inoculated according to the following schedule:

Day	Amount and Site
1	2 ml I.V. (femoral vein) and 0.02 ml I.D. (lower quadrant)
4	2 ml I.V.
11	2 ml I.V.

The course of infection was followed by hematological techniques of peripheral blood study, periodic blood cultures and examination of the blood by dark field illumination.

5. Pathogenicity Study V

Cell suspensions of Strain 400 for purposes of inoculation were prepared by cultivating in a blood-broth medium. The base medium used was Tryptose Phosphate Broth (Difco), supplemented with 10 per cent whole rabbit blood (unhemolyzed). Following an incubation period of 10-12 days, the sedimented intact blood cells were separated from the faintly turbid broth and the latte used for inoculation purposes. The course of infection was followed by the procedures given previously.

6. Pathogenicity Study VI

Strain KC-584 was used in this particular study. It had been serially cultivated on blood agar slants employing Trypticase Soy Agar (Difco) supplemented with 15-20 per cent rabbit blood. Since this isolate would only grow in the presence of 5 per cent CO_2, it was considered to be a Strain variant. Cell suspensions of the organism were prepared by washing 7-10 day old cells from numerous blood agar slants and suspended in a sufficient quantity of physiological saline to give a turbidity equivalent to that of McFarland Standard Number 1. The course of infection in the rabbits employed was followed as described priously. The various routes of inoculation were the subcutaneous and intra-testicular routes. In one animal, endotoxin prepared from Herellea vaginicola was given concurrently with viable cells of B. bacilliformis in an attempt to incite an early inflammatory reaction and decrease host response.

7. Pathogencity Study VII

This particular study employed splenectomized and non-splenectomized rabbits as test animals. Strains 700 and 12, both recent isolates, were used. They were cultivated in the semi-solid medium described earlier and the growth obtained in this medium was used for inoculation purposes. The primary disadvantage in using this technique was that we were injecting organisms and agar constituents. However, in a separate study, Proteose Peptone Agar, the peptone employed in this medium, was found to be non-antigenic and di not give a dermal reaction when inoculated into the rabbit.

72 hour cultures were used throughout this study. Each animal received two inoculations, five days apart, one subcutaneously and one intra-peritoneally. Sera of these animals taken periodically throughout this study were tested for agglutinins and precipitins. In addition, intra-dermal skin test doses were given towards the termination of the study as a means of detecting an immunological response.

8. Pathogenicity Study VIII

Strain 12, the most recent isolate, was used in this study employing the rabbit as test animal. The organism was cultivated in the O-L Medium described previously. Following 5 days incu tion at 25° C, the cells were collected via centrifugation and resuspended in sterile, physiological saline to a concentration of 1×10^9 cells per ml. This concentration gave a hemagglutination titer of 1:32 when employing a 0.4 per cent suspension of human O positive erythrocytes. The various schedules and routes of inoculation are presented in the resultant data of this particular study.

9. Pathogenicity Study IX

This study employed the use of the organisms of the same strain, prepared in the same manner, according to the methods of Pathogenicity Study VIII. Splenectomized and non-splenectomized Rhesus monkeys were used. Animal MX-2 was injected with blood-serum taken from a human case of Bartonellosis at the height of a very severe anemia. The subcutaneous, intraperitoneal and intravenous routes of inocculation were employed.

10. Pathogenicity Study X

The organisms and methods of cultivation are those described in Pathogenicity Study VIII. Two to five day old suckling mice were employed in this particular study. The routes of inoculation were the intra-cranial (0.01 ml), intra-peritoneal (0.02 ml) and intra-muscular (0.03 ml) and intra-muscular (0.03 ml). Impression smears of various tissues and minced tissues were examined and cultured for the presence of B. bacilliformis respectively.

11. Pathogenicity Study XI

Organisms and methods of cultivation are those described for Pathogenicity Study VIII. The Golden hampster and guinea pig were utilized as test animals and various routes of inoculation were employed, i.e., intra-testicular, intra-peritoneal and intra-muscular.

12. Pathogenicity Study XII

Plasma of heparinized guinea pig blood was removed and the buffy coat containing variable, small amounts of red blood cells was suspended in a small value of physiological saline. Equal portions of guinea pig liver, spleen and thymus were minced and ground in an additional, small amount of saline and then slightly centrifuged to remove the larger portions of tissue. The supernate was removed, added to the suspension of blood containing a high leukocyte count (buffy coat) and labeled as "Guinea pig RES Antigen." Four injections of this suspension were injected into each of 2 rabbits in increasing doses at 4-day intervals. Two weeks following the last injection, the rabbits were exsanguinated. The sera from these animals were pooled and labeled as "Guinea Pig RES Immune Serum" (REIS).

B. Antigens and Extracts for Immunological and Pathological Studies

1. Viable, Intact Cells

Organisms of various strains were employed in these studies. They were cultivated in the O-L Medium described earlier, collected via centrifugation and resuspended in physiological saline (pH 7.0) to give a final concentration of 1×10^9 cells per ml.

2. Sonically disrupted cells

Resuspended viable cells were disrupted using a Raytheon 10 kc ultrasonic generator for 8-12 minutes. At the end of this time, phase and dark field microscopy revealed maximum breakage of the cells. The resulting preparations were utilized without further treatment.

3. Endotoxin (LPS) Preparations

LPS preparations were prepared according to the method of Ribi, et al (92,93) with personal modifications. Hereliea vaginicola, Strain 7788 (64) and Bartonella bacilliformis, strain 900, were utilized for these preparations. H. vaginicola was cultivated in the following basic salts medium plus 1.0 per cent sodium acetate:

$NH_4H_2PO_4$........ 0.2 per cent
K_2HPO_4 .... 0.7 per cent
KH_2PO_4 .... 0.3 per cent
$MgSO_4 \cdot 7H_2O$...... 0.01 per cent
pH 7.0

Bartonella bacilliformis was cultivated in the Over-Lay medium described earlier. The cells of each particular organism were collected via centrifugation and washed three times with sterile distilled water. Approximately 15 ml packed cells were resuspended in 400 ml sterile distilled water and homogenized in a Virtis mixer for 5 minutes. Eighty ml of ethyl ether was added to the mixture and allowed to react in a separatory funnel overnight. The residual ether was separated from the aqueous phase containing the cellular material. The latter was then centrifuged to slow speed to remove the intact cells and the resultant supernate was treated with $MgCl_2$ to precipitate the lipo-polysaccharide (LPS) complex (75). The precipitate was washed with distilled water, resuspended in EDTA (75) and dialyzed against running distilled water for 48-72 hours. Following dialysis, the material was freeze-dried under vaccum. Just prior to use, a given amount of the preparation was resuspended in a determined volume of sterile phosphate buffered saline to give the desired concentration.

4. Somatic Antigens

The following, various somatic antigens were prepared from B. bacilliformis strain 500. The organisms were cultivated in the O-L medium employing Eagle's salt solution supplemented with 10 per cent rabbit serum as the Over-Lay broth. Cells were washed, resuspended in sterile physiological saline to give a final concentration of 1 x 10^9 cells per ml and distributed to give three quantities of 35-40 ml per quantity. These aliquots were labeled I, II and III, I serving as an "untreated control antigen" (viable, intact cells). Aliquot II was placed in a boiling water bath for 2½ hours after which time the cells were collected via centrifugation and resuspended in formalized saline to the original volume. This particular antigen was labeled "boiled, somatic antigen" and the supernate was labeled "boiled, cell-free extract." The third aliquot was sonically disrupted for 3 minutes, centrifuged and the supernate labeled "sonic derived, cell-free antigen." The sediment was brought up to the original volume with sterile saline and labeled "sonic derived, cellular antigen."

Rabbits were injected in duplicate with each particular antigen, employing 6, increasing doses given every -3 days. A booster, intravenous injection, was given 8 days following the last injection.

VI. Diagnostic Techniques

A. Hematological

The course of infection during the pathogenicity studies was followed by peripheral red and white blood cell counts, differential white blood cell counts, cell morphology and bleeding and coagulation times. The methods employed were of the conventional, recognized techniques in hematology. Prior to inoculations, all animals employed in these studies were subjected to a series of base-line studies so as to give comparative hematologic data.

B. Clinical

Diagnosis of the hematic syndrome was made primarily from examination of peripherial blood. The defined aspects of significance are as outlined:

1. There is always a degree of anemia which rapidly becomes more severe, comparable to severe, hemorrhagic anemia in development.

2. Marked degenerative activity is indicated in both the erythroid and myeloid series.

3. The number of nucleated red cells may be exceedingly high and reticulocytes may increase to 75 per cent in a severe condition. Such hemopoetic activity is also apparent in bone marrow studies.

4. The pathognomic sign of Oroya Fever is the presence of B. bacilliformis in, or on, the erythrocyte. Up to 90 per cent of the red blood cells may be "parasitized" in a severe infection. The organisms may also be seen in the circulating monocytes.

Unlike Oroya Fever, the histoid syndrome is characterized by a distinctive appearance upon gross examination and is readily established upon physical examination. The verruga nodules vary considerably in appearance, but are basically of three types and distinguished according to evolution, shape, situation, size, disposition and other general aspects of the eruption.

C. Histological

Animals succumbing during the pathogenicity studies or sacrificed for described reasons were routinely examined on a post-mortem basis. Tissue sections of various organs were fixed in 10 per cent neutral formalin and processed for staining. Giemsa's stain for Rickettsia and the Hematoxylin and Eosin histological techniques were employed according to the methods presented in the Manual of Histologic and Special Staining Techniques (61).

D. Bacteriological

When isolating B. bacilliformis from an infected human or animal, two methods were used. One employed inoculating a small portion of aseptically withdrawn blood which had been allowed to clot, into the semi-solid medium. The other method employed inoculating approximately 5 ml of aseptically withdrawn blood directly into the following medium, dispensed in 25-30 ml quantities:

Bacto Tryptose (Difco)..........	10.0 gm
Sodium Chloride.................	5.0 gm
Beef Extract (Difco)............	10.0 gm
Sodium Citrate..................	30.0 gm
Distilled Water.................	1 liter

pH 7.6-7.8

The above techniques have been used with success when isolating the organism from infected humans. However, if the infection is complicated with another organism, the process of selection for B. bacilliformis is to no avail.

VII. Survival Studies

The foremost problem in investigations of this nature is developing a suitable method for the preservation of a maximum number of viable cells for a long period of time. Of the vaious methods used for such, lyophilization, freezing and conventional storage techniques were employed in this study.

The various suspending media used are listed as follows:

1. Defibrinated rabbit blood
2. Skimmed milk
3. Phosphate buffer[1]
4. Phosphate buffer containing 15 per cent glycerol[2]
5. Semi-solid medium (described earlier as growth medium)
6. Rabbit serum
7. Physiological saline containing 0.1 per cent sucrose

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[1]Phosphate Buffer (pH 7.0) M/15

KH_2PO_4...............	79.0 mg
K_2HPO_4...............	100.0 mg
Distilled water......	100 ml

[2]To prepare the above buffer containing 15 per cent glycerol, 15 ml of glycerol were added to the above ingredients and q.s. to 100 ml with distilled water.

Various strains were cultivated, cells collected and washed via centrifugation, resuspended in the various diluents and transferred into lyophilizing vials. For lyophilization, vial suspensions were shell frozen in an acetone bath at approximately -70° C prior to drying. Following lyophilization, the vials were duplicately stored at -70° and -20° C. Vials were re-opened and transferred to the semi-solid medium at various intervals. Additional vial-suspensions were placed at -70° and -20° C and subsequently transferred to the growth medium at various intervals as described above.

In an additional study, various strains were cultivated and stored in the semi-solid medium at -20°, 4° and 28°C. At various intervals, transfers were made to fresh semi-solid media, incubated at 28° C and subsequently checked for viability.

RESULTS

I. Morphological

A. Microscopic

Bartonella bacilliformis stained poorly or not at all with all aniline dyes tested. Therefore, the use of Gram's stain for differential identification and re-evaluation purposes did not prove to be feasible. In addition, no organisms were discernible when stained by the acid-fast technique. The Congo-Red-HCl negative stain revealed the organism to be bacillary to cocco-bacillary in morphology. Such morphology was consistent, non-dependent upon the age of the culture or media used for cultivation purposes. Dark-field illumination microscopy was found to be preferential to phase microscopy when studying the organism in a viable state. The organism demonstrated a somewhat typical appearance with a characteristic motility pattern. In comparison to other organisms, *B. bacilliformis* is of definite, dimunitive size and a cell shape is not readily discernible; the free individual showing as a mere point of activity. The organisms may aggregate into masses which move slowly while the individual cells move more rapidly across the field.

When cultivated in the agar-slant-tissue culture medium, the organism appeared as individual cells and were easily detected with Giemsa's stain. Distinct cocco-bacillary forms were most prominent with coccoid forms being encountered infrequently. In other media, i.e. semi-solid and over-lay, the organisms tended to grow and/or aggregate in masses discernible as colonies. Giemsa's stain was found not to be of value in depicting morphological characteristics of the organism when cultivated thusly. Dark-field illumination proved to be of more value in such instances.

B. Colonial Morphology

Isolated colonies, after 7-10 days incubation on the blood agar plates, were approximately 1.0 mm in diameter, circular, convex, entire, smooth and dark gray in color. Individual colonies were difficult to transfer in light of their being somewhat embedded in the agar surface. Hemolysis was never demonstrated. Micro-colonies were observable within 4 days. Colonies on the infusion agar could be partially washed from the plate. The resulting organisms were constant in their morphology, non-dependent upon the age of the colony beyond 10-12 days. Motile forms were an exception.

II. Cultural and Physiological

a. pH, Temperature and Oxygen Requirements

In the over-lay medium, *B. bacilliformis* grew in colonies of colloidal size on the agar surface at the inter-face of the agar and broth. Such colonies were easily removed by swirling the flask. A complement of experiments were performed using the over-lay medium and varying the temperature, pH and atmosphere of oxygen. No growth was obtained when inoculated flasks (screw-cap type) were incubated at variable temperatures, i.e., 20°, 30°, 37° and 42° C, with the pH being 7.6-7.8. However, optimal growth was obtained when inoculated flasks (screw-cap type) were incubated at 25-28° C, with the pH being 7.6-7.8. Under these same, latter conditions, but using cotton-stoppered flasks, growth was obtained but did not compare with that obtained in stoppered flasks. When the pH of the medium was varied, i.e., 6.0, 6.8, 7.2, 7.4, 8.5 and 9.5, using stoppered flasks, little growth was obtained and was delayed.

It was concluded that for optimal growth in the O-L Medium, it is advantageous to use screw-capped flasks, the pH of the medium should be 7.6-7.8 and the optimal temperature was found to be 28° C.

When employing the semi-solid medium and determining the optimal pH, temperature and oxygen atmosphere for growth, similar results were obtained. In preliminary studies, 2-3 ml of medium was dispensed into 13 x 100 mm tubes. Growth was scant, occurring just below the surface of the medium. However, when 5-6 ml of medium was used, more growth was obtained and occurred below the surface but in discrete, micro-aerophilic bands. Such findings suggested that when more medium was used, a series of more liquid surfaces is provided, at intervals, due to the gradient settling of the medium with time. Bands of growth numbering up to 10, within a depth of 15-20 mm following 4 weeks incubation have been demonstrated. Growth in this medium is also dependent upon a screw-capped or rubber-stoppered tube.

An additional complement of experiments were performed using the heart infusion agar supplemented with defibrinated rabbit blood and glycerol. The temperature, oxygen atmosphere and humidity of incubation were varied. No growth was obtained when the plates were incubated at variable temperatures, i.e., 20-42° C, under 5 per cent CO_2 with the exception of strains KC583 and KC584. When the plates were incubated aerobically, only scant growth occurred. However, if the plates were sealed, optimal growth was obtained between the temperatures of 25-28° C. Under these latter conditions, Strains KC583 and KC584 did not grow and with the other strains, growth was poor and delayed if the temperature was above 28° C.

From these studies, the following comments are made regarding the re-evaluation of the genus <u>Bartonella</u>:

1. In culture, the organism is a cocco-bacillus, usually less than 1.0 microns in the greatest dimension.
2. They do not stain with any of the common aniline dyes nor with the acid-fast stain. They stain with Giemsa's stain following methanol fixation.
3. They are motile, demonstrating a motility visible by dark-field illumination indicative of unipolar, lophitrichous flagella.
4. The organism grows in solid, semi-solid and an over-lay media when the pH is 7.6-7.8, temperature is 28° C and when micro-aerophilic conditions are provided.

B. Nutrient Requirements

The aforementioned media suggest that B. bacilliformis is a fastidious organism, requiring a blood product(s) for growth. However, other factors must be taken into consideration in order to varify such evidence, e.g., (1) are the X and/or V factors solely responsible for growth, (2) is the requirement for a blood product(s) that of nutrition, lending a detoxifying agent(s) to the medium, stabilizing or buffering the environment or providing a given viscosity conducive for growth and (3) are certain enzymes being afforded to the organism through the use of a blood product(s).

Considering these factors, a battery of experiments were designed employing various basal media which were supplemented with a variety of additives. The additives used were in direct concern with establishing the growth requirements of the organism and of replacing the blood product(s) routinely used in the various media.

It was first determined that no contaminants were present in Bacto-Agar (Difco), which could lend nutritive value to the semi-solid medium. The Bacto-Agar formulated in the medium was replaced by Purified Agar (Difco) or Ion Agar Number 2 (Oxoid). In either instance, both media gave good growth of the organism through 7 transfers.

The other constituents of the semi-solid medium, i.e., glucose, serum and hemoglobin solution, were varied in a complement of experiments to provide a more definitive basis of nutrient requirements. The following is a list of the media employed for these purposes, all containing the same concentration of the basal medium and containing pooled serum and/or hemoglobin from the same animals but in varying concentrations:

Basal Medium

Proteose Peptone No. 3	12.0	gm
Bacto Agar (Difco)	13.5	gm
Sodium Chloride	3.0	gm
Distilled Water (pH 8.0)	600	ml

pH 7.3-7.5

Additives		Results (5 days incubation at 28° C)
Glucose	.33*	Negative
Serum	10	
Hemoglobin**	2	
Glucose	.25	
Serum	10	Positive
Hemoglobin	2	
Glucose	.16	
Serum	10	Positive
Hemoglobin	2	

*concentration in per cent

**stock solution prepared by adding 1 part defibrinated rabbit blood to 3 parts sterile, distilled water

Additives		Results
Glucose	0	
Serum	10	Positive
Hemoglobin	2	
Glucose	.05	
Serum	10	Positive
Hemoglobin	2	
Glucose	.05	
Serum	7	Positive
Hemoglobin	2	
Glucose	.05	
Serum	5	
Hemoglobin	2	Positive
Glucose	.05	
Serum	2	Positive
Hemoglobin	2	
Glucose	.05	
Serum	0	Positive
Hemoglobin	2	
Glucose	.05	
Serum	10	Positive
Hemoglobin	1	
Glucose	.05	
Serum	10	Positive
Hemoglobin	.50	
Glucose	.05	
Serum	10	Positive
Hemoglobin	0	
Glucose	0	
Serum	0	Negative
Hemoglobin	0	

From this experiment, it was apparent that glucose was not a requirement for growth of the organism and in addition, it was observed that a 0.33 per cent concentration inhibited growth. The organism will grow in a medium containing either serum and/or hemoglobin which suggests that a factor necessary for growth is present in blood and/or serum. No growth was obtained in a medium depleted of both constituents.

Employing the described semi-solid medium, various concentrations of certain commercial enrichments were substituted for the serum and hemoglobin constituents. They are listed as follows (Difco products unless specified otherwise):

Supplements A, B and C	Ascitic Fluid
Serum Fraction	Dehydrated Hemoglobin (Fisher)
OADC and ADC Enrichments	Hemin (Fisher)
Albumin (Fisher)	

Supplements A, B, C and the serum fraction supported growth through only 2-3 subcultures. The other enrichments were not conducive to growth of the organism through more than 1 subculture.

Since a liquid medium is preferred in cultivating large quantities of the organism, the majority of experimental procedures employed a broth medium. The basal media used were primarily of four types, three being peptones (enzymatic hydrolysates) and one being a basal salts medium (MiddleBrook 7H9 Broth, Difco), supplemented with various casein hydrolysates, carbon sources and enrichments. The following is a list of the media tested:

1. <u>Tryptose Basal Medium</u>

Bacto-Tryptose (Difco)..............	10 gm
Sodium Chloride.....................	5 gm
Beef Extract (Difco)................	10 gm
Sodium Citrate......................	30 gm
Distilled Water.....................	1 liter

2. <u>Tryptone Basal Medium</u>

Bacto Tryptone (Difco).............	10 gm
Distilled Water.....................	1 liter

3. <u>Basal Salts Medium</u>

MiddleBrook 7H9 Broth (Difco).......	4.7 gm
Na_2HPO_4............................	4.5 gm
Distilled Water.....................	900 ml

4. <u>Proteose Peptone Basal Medium</u>

Proteose Peptone Number 3...........	20 gm
Distilled Water.....................	1 liter

The above basal media were supplemented with the following (various concentrations of each were tested):

Fresh rabbit hemoglobin	Sorbo
Hemoglobin plus Supplement C	Methylcellulose
Hemoglobin plus Tween 80	Sucrose
Hemoglobin plus paraffin	Catalase
Hemoglobin plus Supplement B	Edamin
Supplement C	N-Z Amine Type B
Supplement B	Edamin Type S
Tween 80	Ferric ammonium citrate
Paraffin	Sodium citrate
Hemin	Albumin
Defibrinated rabbit blood	Albumin plus catalase
Whole rabbit blood (no anti-coagulant used)	Albumin plus catalase and gelatin
Hemoglobin plus OADC Enrichment	Gelatin
Hemoglobin plus ADC Enrichment	Hemoglobin plus Bacto-Agar extract (boiled)
Hemoglobin plus ascitic fluid	Bacto-Hemoglobin
Fresh rabbit serum	Vitamin free casamino acids
Fresh rabbit serum	Nicotinamide
Cholesterol	Lecithin
Yeast extract	Ascorbic acid
Glutathione	

The results of these cultural studies were discouraging in that relatively few of the media supported growth and in addition, the organism could not be serially transferred in such media. The media which did support some growth through only three transfers are listed below:

1. Liquid Tryptone-Serum or Blood Medium (pH 7.8)

Tryptone Broth	1.5 gm
Fresh rabbit serum or blood	40-50 ml
Distilled Water	150 ml

2. Supplemented Tryptose Phosphate Broth (pH 7.8)

Tryptose Phosphate Broth	1.5 gm
Fresh rabbit serum or blood	20 ml
Distilled Water	100 ml

3. Supplemented Tryptose-Beef Basal Medium (described earlier, pH 7.8)

   Hemoglobin (fresh rabbit), rabbit serum, fresh rabbit hemoglobin plus ADC enrichment and whole rabbit blood supported growth when used as supplements

4. Supplemented Proteose Peptone Broth (pH 7.8)

Proteose Peptone No. 3	2.0 gm
Gelatin (3 percent solution)	20.0 ml
Albumin, Fraction V (35 per cent)	0.3 ml
Catalase	0.5 ml
Distilled Water	100 ml

The information gained from these cultural studies may be summarized as follows:

1. The use of a semi-solid medium supports good growth of B. bacilliformis whereas a broth medium does not, when both are supplemented with fresh rabbit blood products.

2. Albumin and/or catalase cannot replace fresh rabbit blood products in a semi-solid or broth medium.

3. Proteose peptone appears to be the best peptone for use in a basal medium.

4. When considering the role of serum that of rendering a certain viscosity to the medium, it cannot be replaced by Sorbo, methylcellulose, Tween 80 paraffin nor gelatin.

5. Enrichments and supplements (Difco), hemin or dehydrated hemoglobin cannot replace fresh rabbit blood products in a semi-solid or liquid medium.

Subsequent to the development of the O-L Medium, it was attempted to cultivate B. bacilliformis in such a medium substituting the previously described enrichments for the blood products. Through this study, it became apparent that the over-lay technique provided the necessary physical conditions conducive to growth while the fresh blood products could not be substituted to provide the necessary growth factors.

C. Survival Studies

1. Lyophilization and freezing

Initial experiments were carried out to ascertain survival of several strains of B. bacilliformis, lyophilized and frozen in suspensions of skimmed milk, phosphate buffer, phosphate buffer containing glycerol, rabbit serum and physiological saline containing sucrose.

The results of the subcultures for the lyophilization procedure indicated a negative order of survival indicating that the organism is definitely sensitive to freeze-drying. In order to determine whether the freezing or the drying reduced the viability of the cells, the freezing step of the process was repeated using defibrinated rabbit blood, rabbit serum and the phosphate buffer containing 15 per cent glycerol as suspending media. Following the initial freezing at -70° and -20° C, the vials were stored as such for varying lengths of time. The results are shown in Table I.

TABLE I

Results of Survival Studies

Employing Various Liquid Menstra and Frozen at -70° and -20° C

Medium	Time	Results
Skim milk	1 Week	Negative
Buffered Glycerol	1 Week	Negative
Serum	1 Week	Positive
Serum	3 Weeks	Negative
Blood	1 Week	Positive
Bloo[illegible]	3 Weeks	Negative

These results indicate that freezing alone in these particular media has an adverse effect on the organism. Of the four media used, serum and defibrinated blood demonstrated the higher percentages of survival. However, the loss due to freezing is so rapid that complete loss of viability occurs within 3 weeks.

2. Semi-solid menstra

In light of the low survival percentages in lyophilization and/or freezing, an attempt was made to determine if B. bacilliformis could be successfully stored for extended periods of time, at various temperatures, in the semi-solid growth medium. The results of this study are presented in Table II.

TABLE II

Survival of Bartonella bacilliformis at Various Temperatures in a Semi-solid Growth Medium

Temperature	Time in Weeks 1	2	3	4	5	6	7
-20° C	-						
4° C	+	+	-				
28° C	+	+	+	+	+	±	-

This particular medium was shown to afford the highest survival rate when cultures were stored at 28° C, the optimal growth temperature of the organism. Positive subcultures were obtained during 5-6 weeks of storage while negative during the 7th week.

Although negative survival was encountered in the majority of studies utilized, the high survival obtained in the semi-solid menstra demonstrates the success in storing B. bacilliformis cells for the purpose of maintaining stock cultures. It is also indicative of the importance of the temperature and suspending medium in maintaining viable cells.

D. Embryonating Avian Eggs

Jiminez and Buddingh reported that B. bacilliformis can be readily cultivated in vitro and in vivo in the allantoic fluid of 8-12 day embryonating avian eggs and can produce lesions on the CAM (55). In view of such studies, we initiated experimental procedures employing the passage of the organism through embryonating eggs in hope that such treatment would increase the virulence of the organism. On the day egg harvests were done, 1.0-1.5 ml of pooled allantoic fluids were transferred to the allantoic cavities of 7-10 day embryos. At this time, small portions were stained with Giemsa's stain, examined via dark-field microscopy and subcultured to the semi-solid medium. The results are shown in Table III

TABLE III

Data of Serial Transfer of Bartonella Bacilliformis in the Allantoic Cavity of Embryonating Avian Eggs

DAY	NO. EGGS INOCULATED	EMBRYO AGE	TEMP.	GROSS RESULTS	CULTURAL RESULTS	DARK FIELD
1	6	8	37	4 alive	Negative	Negative
	6	8	30	0 alive		
	2 (CAM)	8	37	2 alive (no lesions)		
7	12	8	37	12 alive		
12	24	9	37	16 alive		
16	24	10	37	20 alive		
23	24	7	37	24 alive		

It was apparent from this study that the particular strain of B. bacilliformis with which we were working could not be adapted to embryonated egg cultivation by the methods employed. In all instances, the organism could not be cultivated from yolk material, allantoic fluid nor blood. Embryo mortality was high at lower temperatures. Temperature control embryos indicated that this mortality was due to the temperature and not the organism. No lesions were produced on the CAM as had previously been described.

An additional study was undertaken employing other strains of B. bacilliformis and additional routes of inoculation, i.e., yolk sac and amniotic cavity. At various intervals following injection, egg harvests of each group were subcultured to the semi-solid medium, Giemsa stained and examined via dark-field illumination. The results are presented in Table IV.

TABLE IV

NUMBER OF EGGS*	ROUTE OF INOCULATION	24 hr.	MICROSCOPY AND CULTURAL RESULTS** 48 hr.	72 hr.	7 days
10	Chorio-allantoic membrane	-	-	-	
32	Allantoic cavity	-	-	-	-
20	Yolk sac	-	-	-	-
4	Amniotic cavity	-	-	-	-

* 7-9 day embryonating eggs, incubated at 28° C.
** Harvest material collected at said intervals following day of initial inoculation.

In a separate study, several strains of B. bacilliformis, cultivated in the O-L Medium, semi-solid and solid media, were subcultured into pooled allantoic fluid harvests of normal, uninfected embryonated avian eggs. Such pools were harvested separately from 8, 10 and 12 day eggs. In no instance was growth or viability of the organism demonstrated under such in vitro conditions at 28° or 37° C.

E. Tissue Cultures

In the tissue cultures employing Primary Monkey Kidney, HeLa, HEP2 or Mouse Embryo cells, growth of B. bacilliformis could not be demonstrated. In addition, no cytopathogenic effects (CPE) were demonstrated during a 6-day observation period. After this time period, cell patterns in both the infected and control cultures began to lose their integrity, contributed to normal causes. Identical results were obtained upon 3 subsequent transfers which were performed in an attempt to adapt the organism to the conditions employed. It was concluded that B. bacilliformis does not behave as a facultative intracellular parasite when cultivated in vitro under the given conditions.

In a separate study, the growth medium employed in the tissue cultures was over-layed onto a solid medium according to the techniques described earlier. Under such conditions, growth of the organism was observed. It was therefore postulated that the tissue cells employed did not provide an environment conducive to growth of the organism. Toxic substances, surface active agents and/or certain inhibitory cellular materials of an intrinsic nature apparently inhibited growth.

III. Serological

A. Fluorescent Antibody Technique

Twelve strains of B. bacilliformis examined were stained with the conjugated anti-serum. A group of heterologous Gram negative organisms, i.e., Paracolobactrum species, Pasteurella multicida, Achromobacter aerogenes, Moraxella species, Pseudomonas aeruginosa, Proteus morganii, Serratia marcescens, Herellea vaginicola, Mima polymorpha and Haemophilus influenzae were not stained with this conjugate. In addition, the specificity of the reaction was confirmed by (1) the failure to demonstrate fluorescence in smears treated with normal globulin conjugate, (2) the fluorescence inhibition test and (3) the varying intensities of staining observed in the minute clumps to larger clumps and to the individual cells. These results indicate that such conjugates can be useful in the concise identification of the organism from culture and may provide a rapid, specific test for the demonstration of B. bacilliformis in blood, bone marrow and skin biopsies, providing an early and strongly presumptive laboratory diagnosis.

B. Hemagglutination Technique (HA)

It was found that B. bacilliformis possesses the ability to agglutinate red blood cells. Since the organism is an erythrocyte parasite, it, as with certain viruses, may be adsorbed onto the erythrocyte and these attached bacteria may form "bridges" between the red blood cells producing a weak type of cohesion. This hemagglutination was not restricted to any particular type of erythrocyte. Rabbit, monkey, avian, sheep, guinea pig and human O erythrocytes were found to be agglutinated.

The use of this procedure was 3-fold: (1) as an aid to identification, (2) antigen standardization and (3) to determine the relation of the hemagglutinating ability to viability, virulence and pathogenicity, i.e., the possibility of the development of HA-inhibition antibodies. Numerous strains of B. bacilliformis tested were capable of the hemagglutination phenomenon. Therefore, the HA technique could be used as a supplementary tool for identification purposes. Several untreated, whole cell antigen preparations were periodically checked and demonstrated the capacity to maintain their original HA titer over an indefinite period of time. In addition, intact, viable organisms taken at various stages of growth were shown to hemagglutinate red blood cells. Such a property of the supernate of broth cultures was not demonstrated.

Numerous sera taken from animals during the to be described pathogenicity studies were tested for hemagglutination inhibition antibodies. In no instance could such be demonstrated. It was concluded from these studies that the hemagglutinin of B. bacilliformis is not responsible for protection in the various animal species tested and is not related to the virulence biological property of the organism.

C. Agglutination Phenomenon

Employing the anti-sera of animals subjected to experimental pathogenicity studies, it was found that such animals developed antibodies in high titer to washed cell suspensions of homologous and reciprocal strains of B. bacilliformis. Data obtained during Pathogenicity Study VII, described later, demonstrated that such agglutinins develop late in the course of infection and only following renewed contact with the organism, i.e., upon booster injection. In addition, when these animals, prior to demonstrating such antibodies, were injected intra-dermally with a suspension of viable organisms or a sonically derived extract, no demonstrable skin reaction was observed. However, two uninfected, control animals, which were injected intra-dermally in the same manner, developed skin lesions which progressed through necrosis and ultimately healed.

Such data is indicative of the complexity of B. bacilliformis and the importance of its biological activities demonstrated through immunological procedures. Since agglutination titers were not obtained during the early course of infection, additional studies were preformed, examining other antigens which may be protective in nature and consequently being responsible for the asymptomatic experimental infections induced in the rabbit. Such studies are presented below.

D. Immuno-Diffusion Analyses

In the following studies, the immunology and serology of experimental Bartonellosis and Bartonella bacilliformis were examined, respectively. The antigens employed in successful immunizations are as follows:

1. Sonicated cell suspensions
2. Somatic antigen(s) derived from boiled cell suspensions
3. Cell free extract(s) of sonically disrupted organisms
4. Intact, viable cell suspensions

Using a sonicated cell suspension of Strain 500 as antigen against hyper-immune sera of animals (Numbers R-1000, R-999, R-998 and R-996 - Pathogenicity Study VII) which had received "booster" injections of whole, viable organisms, 4-5 bands of precipitation were observed. In addition, immune sera of animals which had been hyper-immunized with a "sonicated cell antigen" (R-607 and R-608) or "cell free sonicated antigen" (R-609 and R-612) gave one band of precipitation with the homologous antigen. Immune sera of animals which had received the "boiled cell" somatic antigen (R-605 and R-606) gave one band of precipitation when tested against a heterologous antigen, i.e., a sonicated cell suspension.

From these stidues, it was apparent that B. bacilliformis possesses precipitinogens which are able to induce antibody formation to these antigens. However, complex responses were only demonstrable upon hyper-immunization procedures followed by "booster," recall injections.

Utilizing these criteria and techniques of analyses, a study was under taken to (1) determine the homogeneity of the antigen-antibody systems of various strains, (2) enumerate the minimum number of antigens present in each strain and (3) determine if B. bacilliformis possesses common antigen(s) with heterologous Gram negative organisms.

Undiluted hyper-immune anti-sera and sonicated cell suspensions were found to be of optimal concentration for use in the immuno-diffusion analyses. In almost all instances, the sharpest reactions were obtained after 3-4 days incubation in the cold (4° C). Prolonged incubation resulted in diffuse bands that obscured interpretation.

The strains of organisms used in these studies are given in Table V. For simplicity of presentation, we will refer to the various antigens (AG) and anti-sera (AB) of and to particular strains, respectively, by the key numbers in the right hand column.

TABLE V

STRAINS OF BARTONELLA BACILLIFORMIS EMPLOYED IN THE DESCRIBED SEROLOGICAL STUDIES

Code Number	Original Strain Number	Key Designation
500	VA-320	1
12	L-1	2
11	J-27	3
200	JU	4
900	214	5
13	372	6
700	C-2	7
14	VS-15	8
300	157	9
10	172	10
15	208	11
600	C-1	12
17	151	13
400	020	14

Sonicated cell suspensions of the following organisms were employed in the heterologous studies: Bacterium anitratum, Haemophilus parainfluenzae, Haemophilus influenzae, Serratia marcesens, Mima polymorpha, Herellea vaginicola, Moraxella non-liquefaciens and Moraxella liquefaciens.

When homologous studies were done employing antigenic extracts (sonically derived) of B. bacilliformis strains 1, 2, 9, 10, 11, 12 and 15 and their corresponding immune sera, 3-4 distinct antigen-antibody systems were observed. However, when reciprocal analyses were done, only 2-4 antigen-antibody systems were discernible, demonstrating at least one specific antigenic constitutent for some of the particular strains.

More extensive reciprocal analyses were performed to determine the distribution of strain specific antigens. In these studies, two hyper-immune anti sera were employed (AB 1 and AB 2) which demonstrated a more fully complex antibody complement than the other anti-sera. Sonicated cell suspensions of the various strains were employed as antigens. Table VI summarizes the results of this study.

TABLE VI

RECIPROCAL ANALYSES OF VARIOUS STRAINS OF BARTONELLA BACILLIFORMIS

ANTIGENIC EXTRACTS

		1	2	3	4	5	6	7
ANTI SERA	1	4*	3	0	4	4	4	6
	2	3	4	0	3	3	2	6
		8	9	10	11	12	13	14
	1	3	3	4	3	4	4	3
	2	4	3	5	5	4	5	3

* Number of precipitin bands.

From this study it is more apparent that strain specific antigens do exist. However, serological relationships among the organisms are definitely existant. Strain J-27 (key number 3) appears to be in an undefined category.

When the sonicated cell suspensions of the heterologous Gram negative organisms were analyzed against anti-serum 1, a single band of identity was observed in all instances. In addition, four bands were observed with Haemophilus parainfluenzae and Serratia marcesens. To determine if the common line of identity represented the "O" antigen and its antibody, the sonicated cell suspensions were analyzed against anti-serum 2. This particular anti-serum was prepared with an organism shown to be devoid of the "O" antigen as demonstrated in the Immuno-Pathogenicity studies to be described later. When such a system was applied, no band of identity was detected.

IV. Pathogenicity

A. Pathogenicity Study I

It was decided to initiate our study of the animal pathogenicity of B. bacilliformis with the utilization of rabbits as test animals. Rabbits were chosen for four reasons: (a) ease of handling, (b) familiarity with the techniques involved in rabbit inoculation, (c) possibility of developing immune sera for use in later experiments, and (d) observation of the effects produced; the objective being to obtain information as to which clinical procedures to use in following the course of the disease. The rabbits used were numbered consecutively from 100 to indicate the strain employed, preceded by an "R" to indicate animal type, i.e., R-101, R-102 and R-103. All animals were of apparent healthy constitution prior to inoculations.

On the 5th and 6th days of the inoculations, R-102 was noted to apparently have some type of bleeding or coagulation dysfunction, manifested by a markedly long bleeding time following injection. Other than some degree of irritability, R-101 was apparently normal. Blood, taken by cardiac puncture from R-101 and R-102 on the 7th day following the initial injection, showed, on dark-field examination, extremely motile forms morphologically resembling B. bacilliformis. Cultures of this blood were considered positive, the organisms morphologically and culturally resembling B. bacilliformis. R-102 still manifested a haemorrhagic tendency on cardiac puncture. Cultures on R-103, the control animal, were negative. R-101 developed the tendency to haemorrhage on cardiac puncture on the 9th day following the initial injection. R-101 and R-102 appeared to have some degree of anemia; the percentage of cells to plasma being approximately 30 per cent as compared to a 60-70 per cent normal. Morphological examination of blood smears, on both injected rabbits, showed marked anisocytosis, poikilocytosis and polychromatophilia, mild hypochromia, appeared to be thrombocytopenic and a small number of inclusions, characteristic of those found in Oroya Fever, were observed on or in the erythrocytes. R-101 appeared to have leukopenia whereas R-102 appeared to have keukocytosis. Shortly after this time, there was a generalized infection in our entire rabbit population, manifested by positive blood cultures, and due to the confusing clinical conditions so induced, it became necessary to dispose of the animals. R-101 was exsanguinated and autopsied. The pleural cavity showed evidence of haemorrhage with the membrane having petechiae and congested areas. The cavity itself contained a considerable quantity of clotted and autolyzed blood. The chest and thigh dermal layers showed areas of petechiae. Lungs and viscera appeared normal. No histological studies were performed.

Thus, the preliminary pathogenicity studies seemed to indicate that some degree of infection did occur in two rabbits duplicately inoculated. This infection was manifested by: (a) some type of bleeding or coagulation malfunction causing a haemorrhagic tendency, (b) apparently abnormal peripheral blood studies and (c) positive blood cultures. These manifestations suggested that peripheral blood studies, including bleeding and coagulation tests and blood cultures would be most helpful in following the course of infection.

B. Pathogenicity Study II

The second series of pathogenicity studies employed the rabbit as test animal. Prior to inoculations, all animals employed in this study were subjected to a series of base-line studies so as to give comparative hematologic data. The normal data on all animals, presented in Tables VII and VIII corresponded with the findings reported by other workers (135). Tables IX through XI present the findings obtained during this study.

One animal (R-104) expired during the study. The following is the gross and microscopic autopsy data:

Gross examination: No signs of external bleeding were existant. Rigor was present, animal estimated to have expired 5-6 hours before discovery. The chest and thigh regions demonstrated areas of hemorrhage. The liver had an abnormal appearance with hemorrhagic areas centralobularly. Spleen appeared enlarged and congested. Areas of hemorrhage were persistent in the lungs. All other viscera appeared essentially normal.

Bacteriologic examination: Blood cultures taken one day previously and blood from cardiac puncture at autopsy revealed Gram negative bacilli with cultural and biochemical characteristics of *Escherichia coli*.

Miscroscopic examination:

Liver. Two microscopic sections of the liver were examined. There was considerable autolysis with loss of cellular details and diffuse granular change in the cytoplasm. The general architecture of the liver tissue appeared well preserved. No foci of necrosis or other destructive changes were noted. The central veins appeared mildly dilated and in the portal triads there were moderate number of lymphocytes and eosinophils. It was felt that the chronic inflammatory infiltrate was of no diagnostic significance.

Lymph Node: A section of lymph node surrounded by fat was seen adjacent to some fragments of pancreatic parenchyma. The lymphoid follicles were essentially normal. A mild degree of reticular endothelial hyperplasia was noted in some areas. There was no evidence of acute inflammation.

Pancreas: The fragments of pancreas were normal except for autolytic changes.

Thymus: Thymic tissue was seen with some adipose tissue. Some areas of the thymus were autolyzed while others were well preserved. Hassal's corpuscles were observed to be intact, with a few plasma cells seen within the lymphatic tissue. There was no evidence of stress atrophy.

Spleen: There was congestion of the red pulp and mild hyperplasia of the reticular endothelial cells. In many of these great numbers of brownish crystalline

TABLE VII

NORMAL HEMATOLOGIC DATA OF THE RABBITS STUDIED

RED BLOOD CELLS				WHITE BLOOD CELLS			
Determination	Count in millions per c.mm	$(x-\bar{x})$	$(x-x)^2$	Determination	Count in thousands per c.mm	$(x-\bar{x})$	$(x-\bar{x})^2$
1	6.35	.97	.94	1	6.8	0	0
2	6.28	.9	.81	2	8.4	1.6	2.56
3	6.11	.73	.53	3	6.2	.6	.36
4	5.49	.11	.12	4	8.4	1.6	2.56
5	4.86	.52	.27	5	6.2	.6	.36
6	5.55	.17	.29	6	7.9	1.1	1.22
7	5.97	.59	.35	7	6.7	.1	.01
8	5.18	.2	.04	8	9.7	2.9	8.4
9	5.49	.11	.12	9	6.6	.2	.04
10	4.86	.52	.27	10	5.0	1.8	3.24
11	4.98	.6	.36	11	8.0	1.1	1.22
12	4.85	.53	.28	12	8.4	1.6	2.56
13	5.04	.34	.12	13	5.2	1.6	2.56
14	5.68	.3	.09	14	4.5	2.4	5.75
15	5.90	.52	.27	15	7.6	.8	.64
16	5.10	.28	.78	16	5.4	1.4	1.96
17	5.82	.44	.19	17	4.1	2.7	7.29
18	5.09	.29	.84	18	5.4	1.4	1.96
19	4.03	.35	.12	19	8.8	2.0	4.0
20	5.19	.19	.36				

Red blood cells:

$\bar{x} = 5.38 \times 10^6$
S.D.[1] $= 0.66 \times 10^6$
S.E.[2] $= 0.15 \times 10^6$

Significant Difference[3] $= 0.447 \times 10^6$

Normal $\pm$ 1 S.D. $= 5.38 \pm 0.66$

White blood cells:

$x = 6.8 \times 10^3$
S.D. $= 1.61 \times 10^3$
S.E. $= 0.38 \times 10^3$

Significant Difference $= 1.03 \times 10^3$

Normal $\pm$ 1 S.D. $= 6.8 \pm 1.61$

1. S.D. = Standard Deviation
2. S.E. = Standard Error
3. Significant difference (3 S.E.) indicates the change in individual count which would indicate pathologic response.

TABLE VIII

NORMAL HEMATOLOGIC DATA OF THE RABBITS STUDIED

PLATELETS

Determination	Count in hundred thousands per c.mm	$(x-\bar{x})$	$(x-\bar{x})^2$
1	5.5	.62	.384
2	6.0	1.12	1.223
3	5.1	.22	.484
4	4.4	.48	.230
5	4.5	.38	.142
6	4.8	.08	.006
7	5.8	.92	.844
8	4.0	.88	.774
9	4.2	.68	.464
10	4.9	.02	.004
11	4.5	.38	.142
12	4.8	.08	.006

$\bar{x} = 4.88$
S.D. = 0.654
S.E. = 0.188
Significant Difference = 0.564
Normal ± 1 S.D. = 4.88 ± 0.654

DIFFERENTIAL WHITE CELL COUNTS

Determination	%N	%E	%B	%L	%Mo.
1	54	1	2	41	2
2	25		1	70	3
3	44	1	2	51	2
4	41	1	1	60	3
5	42	4		52	2
6	41	5	2	50	2
7	47	1	2	48	2
8	60	3	1	34	2
9	22			76	2
10	50	2	2	44	2
11	30		3	65	2
12	31	3	2	62	2
13	62		12	30	1
14	32			68	
15	40			50	3
16	30	1	—	64	2
Mean percentage	40.6	1.4	2	54	2

The individual variation in these percentages was so great as to invalidate standard deviation data.

TABLE IX

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 101-b
Animal Type: Rabbit
Antigen Number: 100(520/63)

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Blood Culture
4/22/63	5.45	5.05	91		9			1' - 2'	-
5/21/63	5.32	5.40	88		11		1	1' - 2'	-
5/22/63			72		28			1' - 2'	+
5/23/63	5.37	4.25	70	4	22	3	1	30" - 1½'	+
5/24/63	4.11	9.10						30" - 1½'	+
5/25/63	3.66	8.35	41	6	53			25" - 5'	+
5/27/63	3.86	9.00	70	3	27				
5/31/63	4.48	5.35	60	2	30	5	3	1¼' - 5½'	
6/5/63			61	2	34	3		30" - 7'	-
6/11/63	4.57	6.00						20" - 1'	-
6/18/63	4.62	6.10	58	1	36	4	1	30" - 1'	-

TABLE X

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 102-b
Animal Type: Rabbit
Antigen Number: 100(5/20/63)

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Blood Culture
4/22/63	5.69	[illegible].80	81		19			2' - 2'	-
5/21/63	5.91	[illegible].20	61		38		1	2' - 2'	-
5/22/63			55		38	7		2' - 2'	+
5/23/63	5.50	1[illegible].70	56	3	40		1	20" - 1½'	+
5/24/63	5.13	1[illegible].00	53		43	1	3	45" - 2'	+
5/25/63	6.24	[illegible].10	43		54		3	40" - 1½'	+
5/27/63	4.42	[illegible].05	59		42				
5/31/63	4.97	[illegible].30	73	28				1' - 5½'	+
6/ 5/63			60	5	30	4	1	30" - 5'	-
6/11/63	5.28	6.50							
7/18/63	5.42	5.80	65	2	30	3		20" - 8"	-

TABLE XI

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 104
Animal Type: Rabbit
Antigen Number: 100(5/20/63)

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Blood Culture
4/22/63	5.36	10.2	88		12			1½' - 1½'	-
5/21/63	5.42	6.95	78		22			1' - 1½'	-
5/22/63			71	1	25	3		1½' - 1½'	+
5/23/63	6.12	8.7						1½' - 1½'	+
5/24/63	5.72	12.15						20" - 1½'	+
5/25/63	5.28	9.9	60		40			30" - 2½'	+
5/27/63	4.25	12.4							
5/31/63	4.93	12.5	68		32			30" 0 1'	-

granules averaging 1 to 2 microns in diameter were observed. The lymphoid corpuscles did not present pathologic change.

Heart: The myocardium was essentially normal.

Kidney: A severe degree of autolysis was noted in the section of kidney parenchyma. No acute inflammatory changes were observed.

Lung: The section of lung demonstrated foci of pneumonia, characterized by moderate sero-fibrinous exudation and the presence of many eosinophils. Areas of lymphocytic infiltrate were also observed.

Diagnosis:
1. Pneumonia (etiology undetermined)
2. Congestion and hyperplasia of the spleen
3. Reactive hyperplasia of the lymph nodes
4. Autolysis of the liver and kidneys

The cause of death was not definitely established. Giemsa stains to detect the presence of B. bacilliformis within the tissues were negative.

The third day following injection (May 23, 1963), the blood cells began to deviate from an essentially normal appearance. Some Bartonella-like inclusions were noted and a mild anisocytosis, poikilocytosis and polychromasia were evidenced. There was an occasional atypical lymphocyte, and occasionally the polymorphonuclear leukocytes appeared as very large cells with 6-8 segments per cell. A small percentage of nucleated red blood cells were observed while the platelets appeared adequate. In the following days, the anisocytosis and poikilocytosis remained mild whereas the polychromasia became marked and inclusions became numerous. The nucleated red blood cells increased and there were many nuclear remnants such as Howell-Jolly bodies, Cabot rings, basophilic granulation and marked rouleaux formation was observed. Several of the nucleated red blood cells demonstrated karyohorrexis. The leukocytes demonstrated toxic granulation.

Four days following the end of the inoculation schedule, the appearance of the cell morphology began to revert to normal. Inclusions were fewer, the number of nucleated red blood cells decreased and the majority of the white blood cells were of normal appearance. A mild hypochromia, polychromasia, anisocytosis and poikilocytosis persisted until June 18, 1963. After this time all hematologic studies were normal.

C. Pathogenicity Study III

It was the purposes of this study to (a) attempt giving duplicate comparative results as those found in the second series of study and (b) through the use of agents capable of lowering host resistance and antibody responses, to show more significant pathological changes. The results are presented in Tables XII through XVI. It was apparent that the use of agents capable of reducing host resistance and antibody response did not enhance the pathogenic properties of B. bacilliformis.

TABLE XII

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 201
Animal Type: Rabbit
Antigen Number: 200(7/8/63)

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Platelet Count	Blood Culture
7/12/63	5.97	3.75	48	2	47	1	2	1' - 2'	290,000	-
7/15/63	5.18	5.15	68		32				280,000	-
7/16/63	5.17	7.3						1' - 3'	310,000	+
7/17/63	5.63	5.2	36	1	54	3	5		520,000	+
7/18/63	5.2	5.2						1' - 2'	400,000	+

TABLE XIII

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 202
Animal Type: Rabbit
Antigen Number: 200(7/8/63)

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Platelet Count	Blood Culture
7/8/63	4.86	6.15	52	2	42	4		1' - 1'		-
7/12/63	4.85	5.0							510,000	-
7/15/63		4.1							450,000	+
7/16/63										+
7/17/63	4.32	4.95	64	1	44	2		1' - 2½'	390,000	+
7/18/63	4.94	3.55						30" - 2'	420,000	+
7/19/63	4.76	4.0						3' - 3'	330,000	+

TABLE XIV

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 203
Animal Type: Rabbit
Antigen Number 200(7/8/63)

Agent used to reduce animal resistance and antibody response: ACTH

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Platelet Count	Blood Culture
7/8/63	6.35	6.75	41	2	54	1	2			-
7/12/63	5.18	6.7	34	60	60	3	1	1' - 2'	440,000	-
7/15/63	5.1	4.45	50	2	40		7		400,000	+
7/17/63	4.65	7.65	54	1	43	1	1	45" - 2½'	670,000	+
7/18/63	5.33	4 2						1' - 2'	380,000	+
7/19/63	4.69	6.95						20" - 2½'	980,000	+

TABLE XV

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 205
Animal Type: Rabbit
Antigen Number: 200(7/8/63)

Agent used to reduce animal resistance and antibody response: Mucin

Date	RBC	WBC	D I F F E R E N T I A L					B and C	Platelet Count	Blood Culture
7/8/63	6.28	8.35	70	3	25	1	1		450,000	-
7/12/63	5.49	9.70							420,000	-
7/15/63	4.82	7.60							470,000	+
7/17/63	4.55	10.50	72	1	22	2	3	1' - 4'	480,000	+
7/18/63	4.79	22.60						30" - 1'	470,000	+
7/19/63	3.65	9.20							310,000	+

TABLE XVI

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 206
Animal Type: Rabbit
Antigen Number: (200(7/8/63)

Agent used to reduce animal resistance and antibody response: 6-Mercaptyl Purine

Date	RBC	WBC	DIFFERENTIAL L	M	S	E	B	B and C	Platelet Count	Blood Culture
7/8/63	6.23	6.2	51	2	44	1	2			-
7/12/63	5.49	6.55	44	2	50	2	2		480,000	-
7/15/63	5.82	5.4							490,000	-
7/17/63	3.4	8.25	56	1	40	2	1	20" - 1'	620,000	-
7/18/63	3.6	4.3						1' - 2'	370,000	+
7/19/63	5.65	5.65							710,000	+

On the third day of the inoculation schedule (July 17, 1963), the cell morphology of the peripheral blood cells began to deviate from the normal. There was a mild anisocytosis and poikilocytosis with a moderate polychromatophilia. There were many extremely macrocytic red blood cells which contained from 30-50 basophilic inclusions. These did not have a distinctly bacteria-like morphology and the cause or origin of these inclusions was not known. Such inclusions increased to considerable amounts on the following day. A leukocytosis was evident but this condition corrected its deviation from the normal on the subsequent day. The blood picture did not change drastically from this appearance for the remainder of this analysis.

The platelet count underwent an interesting increase although the bleeding and coagulation times did not deviate essentially from normal as observed in R-201. Such findings were contributed to the initial relatively low platelet count of this animal, which did not ultimately reach the actual normal value expected in the rabbit. Therefore, the bleeding and coagulation times would not necessarily be expected to deviate unless the value exceeded the normal limits expected.

In animals R-202, R-203 and R-206, the platelet count underwent interesting deviations which were paralleled to some degree by the deviations in the bleeding and coagulation times.

D. Pathogenicity Study IV

This particular study employed the Rhesus monkey as test animal. Table XVII presents the normal hematologic data obtained during a series of base-line studies prior to inoculation.

Table XVIII presents the hematologic findings obtained during the course of this study. It may be seen that no significant hematologic changes were observed during the experimental infection. In addition, all blood cultures were negative for B. bacilliformis and no dermal lesions developed at the site of the intra-dermal inoculations. Such data is found to be directly opposite to the findings obtained by other workers when employing the monkey as test animal (see Literature Review).

The study was discontinued when it was found that the animals under study had developed a transient bacteremia, e.i., two Paracolobactrum species (P. intermedium and P. escherichieae) and a Salmonella (Group B) species. The significance of such a condition was not overlooked since it has been reported that secondary inter-current infections occur in approximately 48 per cent of primary Bartonella infections and that the secondary invading organisms may be responsible for the high mortality rate observed in the disease. However, the test animals remained grossly normal and no complications were evidenced. On the basis of the distinctive lack of any significant hematologic changes, negative blood cultures for B. bacilliformis and no dermal lesions, it was concluded that the organism being used was of an avirulent type. In possible correlation, the organism which we employed differed in its physiologic growth requirements as compared to the reported requirements of B. bacilliformis, i.e., the organism grew best under 5 per cent CO_2, growth was not obtainable at temperatures above 25° C, a high humidity was essential and growth could not be obtained without the inclusion of erythrocytes. Such requirements were in contradistinction to the reported aerobic conditions, growth at 37° C and a normal incubator humidity.

TABLE XVII

STATISTICAL NORMAL HEMATOLOGIC DATA OF THE MONKEYS STUDIED*

WBC (in thousands per cubic millimeter):

Mean = 9.145
S.D. = 2.60
S.E. = 0.82
Normal = 9.145 ± 2.60
Significant Difference = 2.46

RBC (in millions per cubic millimeter):

Mean = 5.567
S.D. = 0.53
S.E. = 0.16
Normal = 5.567 ± 0.53
Significant Difference = 0.48

Platelet (in hundred thousands per cubic millimeter):

Mean = 3.2
S.D. = 1.02
S.E. = 0.29
Normal = 3.2 ± 1.02
Significant Difference = 0.87

Differential White Cell Determination (in percentage)

Mean:

Neutrophilis	=	34.6
Lymphocytes	=	58.1
Monocytes	=	4.3
Basophilis	=	0.2
Eosinophils	=	2.5

The individual variation in percentages was so great as to invalidate standard deviation data.

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*S.D. = Standard deviation
S.E. = Standard error

Significant Difference (3 S.E.) indicator. The change in individual count which would indicate pathologic response.

TABLE XVIII

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Initial Inoculation Date: 12/17/63

Organism Injected: Strain 300

Monkey 179

DATE	RBC	WBC	DIFFERENTIAL L	M	S	E	B	PLATELET
12/10/63	5.28	9.25	49	6	41	4	-	300,000
12/12/63	6.13	11.5	63	3	32	2	-	290,000
12/17/63	6.09	10.35	58	4	37	1	-	370,000
12/20/63	5.43	14.0	50	5	43	2	-	
127/27/63	5.43	9.8	64	3	33			330,000
12/31/63	4.97	13.95						290,000
1/3/64	5.09	11.4						250,000

Monkey 73

DATE	RBC	WBC	L	M	S	E	B	PLATELET
12/10/63	4.49	9.8	60	5	30	4	-	360,000
12/12/63	5.76	11.8	57	2	40	1	-	240,000
12/17/63	6.43	7.3	66	4	29	1	-	520,000
12/20/63	5.75	7.1	89	1	10	-	-	
12/27/63	5.43	9.8	80	2	17	-	1	270,000
1/ 3/64	5.26	8.8						580,000

E. Pathogenicity Study V

Previous pathogenicity studies reported here which employed the rabbit as test animal appeared to indicate that a low degree of infection could be established with some strains of B. bacilliformis. Therefore, additional studies were initiated in an attempt to reproduce these results using another strain of the organism and employing previously used and additional modes of inoculation, i.e., I.V., I.P., and intra-testicular (I.T.). All base line results were tabulated and animals showing the least significant deviation calculated on the basis of standard error were used in the study.

Tables XIX through XXI present the findings obtained during the course of the experimental infection. The results obtained gave additional evidence concerning the possibility that we were working with an avirulent strain of B. bacilliformis. This conclusion was primarily based on the fact that these results were not comparable with those obtained in the earlier presented studies, at which time other strains were used.

Other workers have reported a significant, detectable infection comparable to Bartonellosis when using the intra-testicular route of inoculation (107). In the present study, we were unable to induce an infection by this route; even when other modes were combined with the I.T. route.

F. Pathogenicity Study VI

Prior to inoculations, all animals employed in this study were subjected to a series of base line studies so as to give comparative data. These normal and post-infection findings are presented in tabular form in Tables XXII through XXVI.

It can be seen from the results of this study that only the animals inoculated (a) alternately intratesticularly and intravenously and (b) subcutaneously with a mixture of live, viable organisms and endotoxin, demonstrated a form of experimental infection. As compared to the other animals (R-158, R-159, and R-160) such modes of inoculation resulted in a deviation from the normal, characterized by anisocytosis, poikilocytosis and hypochromis. Macrocytic red blood cells containing basophilic inclusions were also observed. However, the inclusions did not have a distinctly bacteria-like morphology. The cause of death of R-161 was not determined and all blood cultures taken during the course of the experimental infections were negative for B. bacilliformis.

G. Pathogenicity Study VII

The course of infection of each animal is presented in tabular form, Tables XXVII through XXXII. Neither precipitins nor agglutinins were demonstrable during the course of infection nor during the convalescent stage. However, when the animals received a "booster" injection of intact, viable organisms, an anamnestic response was seen (in actuality the primary response), precipitins demonstrable within 7 days following the "booster" injection.

TABLE XIX

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 133
Animal Type: Rabbit
Route of Inoculation: I.P.*

Day	RBC	WBC	D I F F E R E N T I A L						Blood Culture
			L	M	S	E	B	H**	
Pre-inoculation	5.6	6.4	59.7	2.0	38.3	0	2	0	-
Post-inoculation									
1	3.8	7.5	55		41		2	2	-
3	3.8	8.0	65	1	34				-
5	3.8	11.2	66	1	31		2		-
8	3.3	6.8	62		38				-
15	4.0	6.0	60	1	39				-

*The animal received increasing doses of live cells, every other day for 8 days. Amounts ot inoculum were 2.0 ml to 8.0 ml

**Heterophiles (classified under the polymorphonucelar cells)

TABLE XX

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal Number: 135
Animal Type: Rabbit
Route of Inoculation: I.V.*

Day	RBC	WBC	DIFFERENTIAL L	M	S	E	B	H	Blood Culture
Pre-inoculation	4.0	7.0	70		20			10	-
Post-inoculation									
1	3.7	7.5	67		27				-
3	3.75	7.8	79	1	19				-
5	4.8	11.0	81	1	15				-
8	4.0	13.35	48	1	51				-
15	4.0	10.0	80		20				-

*The animal received increasing doses of live cells, every other day for 8 days. Amounts of inoculum were 1.0 ml to 8.0 ml.

TABLE XXI

COMPARATIVE HEMATOLOGIC STUDIES DURING COURSE OF INOCULATION

Animal number:
Animal type:
Route of Inoculation: The series of inoculation were as follows: Day 1, 0.5 ml intratesticularly (I.T.); Day 3, 2.0 ml I.V., and 4.0 ml I.P.; Day 5, 8.0 ml I.V., 4.0 ml. I.P. and 0.5 ml. I.T.; Day 6, 4.0 ml. I.V. and 4.0 ml. I.P.

Day	RBC	WBC	DIFFERENTIAL L	M	S	E	B	H	Blood Culture
Pre-inoculation	5.2	6.8	63	2	35				-
Post-inoculation									
1	5.0	7.1	60		27	2	6	5	-
3	4.75	10.8	46	3	48	1	2		-
5	5.0	12.25	79		18		3		-
8	3.6	6.85	17	1	16	1	5		-
15	4.8	6.8	55	2	40	1	2		-

TABLE XXII

Animal No. R-156

Day	RBC	WBC
1	5.50	5.00
2	4.88	8.30
4	4.15	5.95
6	4.10	5.00
6	Animal injected with 0.3 ml organisms intratesticularly	
8	4.50	6.20
8	Animal injected with 0.3 ml organisms intratesticularly	
11	4.70	5.60
11	Animal injected with 1.0 ml organisms intravenously	
15	4.60	8.10
15	Animal injected with 0.3 ml organisms intratesticularly	
19	4.50	9.00
19	Animal injected with 1.5 ml organisms intravenously	
24	3.00	10.00
29	3.60	14.50
33	4.30	10.60
38	4.50	11.00

TABLE XXIII

Animal No. R-158

DAY	RBC	WBC
1	5.08	3.75
2	4.88	8.25
4	5.16	5.05
6	4.50	6.00
6	Animal injected with 1.0 ml organisms subcutaneously	
8	4.70	7.00
8	Animal injected with 2.0 ml organisms subcutaneously	
11	4.10	8.0
11	Animal injected with 4.0 ml organisms subcutaneously	
15	4.30	7.0
15	Animal injected with 8.0 ml organisms subcutaneously	
19	5.0	8.0
19	Animal injected with 8.0 ml organisms subcutaneously	
24	4.70	5.50
29	5.10	5.85
33	4.60	5.80

TABLE XXIV

Animal No. R-159

DAY	RBC	WBC
1	4.85	6.00
2	5.10	5.70
4	4.20	5.55
6	4.00	4.80
6	Animal injected with 1.0 ml organisms subcutaneously	
8	4.00	5.00
8	Animal injected with 2.0 ml organisms subcutaneously	
11	4.40	5.00
11	Animal injected with 4.0 ml organisms subcutaneously	
15	5.00	4.50
15	Animal injected with 8.0 ml organisms subcutaneously	
19	4.40	8.65
19	Animal injected with 8.0 ml organisms subcutaneously	
24	5.10	5.50
29	5.30	7.00
33	5.25	4.75
38	5.00	4.95

TABLE XXV

Animal No. R-160
Animal Type: Control

DAY	RBC	WBC
1	4.5	4.25
2	5.6	4.50
4	4.53	3.75
6	3.80	4.25
6	Animal injected with 0.6 mg endotoxin I.V. (0.3 ml)	
8	3.90	6.0
8	Animal injected with 0.6 mg endotoxin subcutaneously (0.3 ml)	
11	4.10	4.5
15	4.60	5.0
15	Injected with 0.6 mg endotoxin subcutaneously (0.3 ml)	
19	4.40	4.0
24	4.75	3.5
29	4.00	6.0
33	4.50	6.25
38	4.60	3.50

TABLE XXVI

Animal No. R-161

DAY	RBC	WBC
1	5.87	5.8
2	4.85	4.25
4	4.53	4.15
6	4.70	5.50
6	Animal injected with 0.6 mg endotoxin mixed with 1.0 ml viable organisms subcutaneously	
8	4.40	3.80
8	Animal injected with 0.6 mg endotoxin mixed with 2.0 ml viable organisms subcutaneously	
11	4.60	5.50
11	Aniami injected with 4.0 ml viable organisms subcutaneously	
15	4.70	4.50
15	Animal injected with 0.6 mg endotoxin mixed with 5.0 ml viable organisms subcutaneously	
19	4.60	4.00
19	Animal injected with 8.0 ml viable organisms subcutaneously	
24	4.60	3.00
29	4.60	6.25
33	2.40	6.25

TABLE XXVII

Animal No. R-1000
Animal Type: Rabbit (splenectomized)

Day	RBC	WBC	Differential S	L	M	E	B	Blood Culture	Serology
1	5.33	7.05	Not done					negative	negative
3	4.75	10.15	Not done						
6	4.25	8.10	Not done					negative	
8	4.89	8.70	33	64	2	0	1		
8	Animal injected with 2.0 ml viable organisms subcutaneously								
13	5.19	7.45	39	55	3	1	2		
13	Animal injected with 3.0 ml viable organisms intraperitoneally								
17	4.93	8.55	32	61	6	0	1	negative	
24	5.13	14.90	37	60	1	0	3	positive	negative
29	4.91	11.20	54	41	5	0	0	positive	
35	5.44	18.30	53	42	3	1	1	positive	
43	5.30	11.30	45	52	1	0	2	negative	
65									negative
78	Animal injected intradermally with viable organisms (several sites) No dermal reactions were observed								
107									negative
111	Animal was injected with 2.0 ml viable organisms (strain 500)								
118									positive

TABLE XXVIII

Animal No: R-999
Animal Type: Rabbit (splenectomized)

Day	RBC	WBC	Differential S	L	M	E	B	Blood Culture	Serology
1	5.09	4.40	Not done					negative	negative
3	5.04	8.35	Not done						
6	4.38	8.75	Not done						
8	4.69	9.10	34	62	0	0	4		
8	Animal injected with 2.0 ml viable organisms subcutaneously								
13	5.22	7.50	38	58	2	0	2		
13	Animal injected with 3.0 ml viable organisms intraperitoneally								
17	5.32	8.25	33	65	1	0	1	negative	
24	5.77	11.05	43	55	1	0	1	positive	negative
29	4.86	12.30	42	56	0	0	1	positive	
35	5.86	13.15	34	63	2	0	1	positive	
43	5.30	11.50	35	56	4	2	3	negative	
65									negative
111	5.37	10.00	35	61	4	0	0		
111	Animal injected with 2.0 ml viable organisms subcutaneously (strain 50C)								
118									positive
129	5.81	12.05	Note done						

TABLE XXIX

Animal No: R-998
Animal Type: Rabbit (splenectomized)

Day	RBC	WBC	Differential S	L	M	E	B	Blood Culture	Serology
1	4.98		Not done					negative	negative
3	4.30	8.85	Not done						
6	4.40	7.95	Not done						
8	4.43	11.35	34	63	8	0	0		
8	Animal injected with 2.0 ml viable organisms intraperitoneally								
13	4.77	11.15	27	68	4	0	1		
13	Animal injected with 3.0 ml viable organisms subcutaneously								
17	4.82	7.90	39	58	2	0	0	negative	
24	4.59	8.65	44	52	2	0	2	negative	negative
29	4.73	12.25	46	54	0	0	0	positive	
35	4.63	12.35	38	59	2	0	1	positive	
51	5.11	11.15	Not done						
65									negative
71	Animal inoculated intradermally with "borken cells" (sonically derived) No dermal reactions were observed								
107									negative
111	5.65	11.90	49	50	1	0	0		
111	Animal injected with 2.0 ml viable organisms (strain 500)								
118	5.58	8.65							

TABLE XXX

Animal No: R-996
Animal Type: Rabbit (non-splenectomized)

Day	RBC	WBC	Differential S	L	M	E	B	Blood Culture	Serology
1	4.80	4.50	Not done					negative	negative
6	4.35	6.30	Not done						
8	4.29	7.65	27	66	7	0	0		
8	Animal injected with 2.0 ml viable cells intraperitoneally								
13	4.73	5.50	38	59	0	0	3		
13	Animal injected with 3.0 ml viable cells subcutaneously								
17	4.16	6.35	42	58	0	0	0	negative	
24	5.20	5.20	33	65	0	0	2	negative	negative
29	4.85	5.75	38	61	1	0	0	negative	
35	6.00	9.00	40	56	4	0	0	negative	
43	5.25	12.00	31	65	0	1	3	positive	
51	5.06	6.40	Not done					negative	
65									negative
78	Animal inoculated intradermally with "broken cells" (sonically derived), uninoculated media and viable organisms								
107		No dermal reactions observed							negative
111	4.95	9.15	49	47	4	0	0		
111	Animal injected with 2.0 ml viable cells intraperitoneally								
118									positive
129	5.33	9.15							

TABLE XXXI

Animal No: R-995
Animal Type: Rabbit (Control)

Day	RBC	WBC	Differential S	L	M	E	B	Blood Culture	Serology
1	4.75	3.25	Not done					negative	negative
3	4.84	3.50	Not done						
6	4.25	3.65	Not done						
8	4.50	4.00	33	61	2	0	4		
8	Animal injected with 2.0 ml uninoculated medium intraperitoneally								
13	6.00	4.65	25	67	3	1	4	negative	
17	5.21	5.70	41	56	3	0	0	negative	
24	5.04	5.65	44	55	0	1	0	negative	negative
29	5.11	6.30	38	61	1	0	0	negative	
35	5.24	5.60	43	53	3	0	1	negative	
43	5.12	5.90	47	46	2	0	3		
50	Animal injected intradermally with viable organisms (strain 500) Dermal reactions observed after 48 hrs.								
71	Animal injected intradermally with "broken cells" (strain 500) Dermal reactions observed after 48 hrs.								
107									negative
111	5.52	5.75	55	43	2	0	0		
111	Animal injected with 2.0 ml viable organisms (strain 500)								
118									negative
129	5.93	7.50							

TABLE XXXII

Animal No: R-994
Animal Type: Rabbit (Control)

Day	RBC	WBC	Differential S	L	M	E	B	Blood Culture	Serology
1	4.70	5.85	Not done					negative	negative
3	4.45	8.30	Not done						
6	4.55	7.45	Not done						
8	4.35	6.15	48	50	1	0	1		
8	Animal injected with 2.0 ml uninoculated medium subcutaneously								
13	4.79	5.70	38	60	1	0	1	negative	
17	5.21	8.15	27	72	1	0	0	negative	
24	4.58	8.05	24	75	1	0	0	negative	negative
29	5.53	9.25	27	72	2	0	0	negative	
35	5.10	9.50	30	66	1	0	3	negative	
43	5.00	11.30	22	74	3	0	1	negative	

We concluded from these studies that an asymptomatic infection was induced in all animals receiving doses of intact, viable organisms. Such an assumption is based on the following findings:

(1) A detectable, relative leukocytosis observed following an incubation period of 18-25 days with an increase in the segmented neutrophils.
(2) Positive blood cultures paralleling the relative leukocytosis.
(3) Bartonella-like inclusions observed in or on the red blood cells.
(4) Anisocytosis, poikilocytosis and a mild hypochromia without a detectable decrease in the red blood cell count.
(5) A detectable anamnestic response in all animals.
(6) The demonstration of negative dermal reactions when the animals were injected intra-dermally during the convalescent stage, with viable cells, while control animals developed dermal reactions.

H. Pathogenicity Study VIII

This particular study employed the rabbit as the test animal. Due to the various schedules and routes of inoculation, each individual animal will be discussed separately for convenience. The following data sheets present the findings obtained:

Rabbit Number 172: A series of preinfection hematological base-line studies revealed the animal to be in a state of apparent good health. The weight of the animal at this time was approximately 10 pounds. On day 1, 0.6 ml of a viable cell suspension was injected into each testicle and 0.5 ml intravenously. On day 3, the right testicle was noted to be extremely hemorrhagic and enlarged. On this date the animal was again injected intra-testicularly (0.6 ml into each testicle) and intravenously (1.0 ml). The orchitis involving only the right testicle was noted to be progressive with an evident necrosis of the scrotal sac. The animal was irritable, apathetic and a weight loss of 3 pounds was observed. On day 6, the animal was injected I.V. with 2.0 ml viable organisms. The animal was reported to have lost 5 pounds on this date. The animal was found dead on day 8, death having occurred approximately 24 hours prior to the finding of the animal. The only external signs visible were those of hemorrhage from the nose and a dark skin suggesting internal hemorrhage. In addition, the left testicle was noted to be hemorrhagic and enlarged. Post-mortem findings are as follows:

Liver: Enlarged, hemorrhagic necrosis suggestive of acute congestion, capsular areas resembling anemic infarcts, gall bladder intact.

Testicles: Right testicle extremely hemorrhagic, with necrotic areas existent. Left testicle slightly hemorrhagic.

Axial Lymph Nodes: Slightly enlarged, hemorrhagic.

Spleen: Extremely hemorrhagic with evident necrosis, atrophied, (congestive).

Heart: Hemorrhagic, extremely soft, enlarged, capsular areas resembling infarction and fibrotic in appearance.

Kidneys: Extremely hemorrhagic, black in appearance, kidney-bean morphology absent.

Lung: Pulmonary infarcts with necrosis foci of consolidation appearing as pale and gray patches, areas of hemorrhagic and granulomatous-like lesions.

Rabbit No. 619: A series of pre-infection hematological base line studies revealed animal to be in good health. The animal was inoculated as follows using a "boiled cell suspension" of B. bacilliformis and the intravenous route of inoculation:

Day 1 - 0.2 ml
Day 3 - 0.4 ml
Day 6 - 0.8 ml
Day 9 - 1.5 ml
Day 13 - 2.0 ml

The animal expired immediately following the last inoculation. The symptoms accompanying death were those of anaphylactic shock. The preliminary reaction consisted of irregular respiration, succeeded by panting. The ears became hyperemic and then blanched. The animal collapsed, laid out-stretched, gave a few convulsive movements and died suddenly with the head thrown back and eyes protruding. Upon autopsy, the right side of the heart was found greatly dilated and the inferior vena cava, protal vein and liver were, literally, engorged with blood.

We were unable to produce this shock in a second animal, R-620, inoculated in the same manner as R-619. However, recalling the mechanism of active anaphylaxis, it takes place in an animal which is first sensitized by injection of antigen and is later shocked by reinjection of the same antigen. R-619 evidently responded to a series of injections rather than a single injection leading to the sensitized state whereas R-620 was not sensitized through the series of injections. Further investigations are in process to substantiate these findings.

Rabbit No. 617: A series of pre-infection hematological base line studies revealed the animal to be in good health. The weight of the animal at this time was 4 pounds. The animal received the following injections:

Day 1 - 0.6 mg endotoxin mixed with 1.0 ml organisms and given sub. cu.
Day 3 - 0.6 mg endotoxin mixed with 2.0 ml organisms and given sub. cu.
Day 6 - 4.0 ml organisms given sub. cu. (weight of the animal 2½ lbs.)

The animal expired on Day 9. External bleeding was observed from the nose. Semi-rigor present, animal estimated to have expired 5-6 hours before discovery. Areas of hemorrhage on the chest, ear and thigh region. Post-mortem findings are as follows:

Heart: Extremely soft and hemorrhagic. Appeared fibrotic and glassy.

Lung: Pulmonary infarcts with areas of necrosis, foci of consolidation appearing as anemic-infarcts.

Kidneys: Extremely dark and hemorrhagic.

Spleen: Hemorrhagic and atrophied.

Thymus: Degenerated and hemorrhagic.

Liver: Essentially normal, gall bladder intact. A few areas of hemorrhage, no necrosis.

Rabbit No. 120 was inoculated in the same manner. However, this animal was splenectomized. Death did not occur in this animal nor does it show signs of experimental infection on this date.

Rabbit No. 160: A series of pre-infection hematological base line studies revealed the animal to be in good health. The weight of the animal at this time was 10 pounds. On Day 1,injected with 0.3 ml viable organisms into each testicle (I.T.) plus 0.5 ml intravenously (I.V.). On Day 3, the animal was injected with 0.4 ml viable organisms into the left testicle plus 1.0 ml I.V. A third inoculation was given on Day 6, 2.0 ml I.V. On Day 8, the animal weighed 6 pounds, was extremely apathetic, hyper-ventilated, near death and sacrificed. The external signs visible were those of an exudative material being secreted from the nose and a hemorrhagic appearance of both testicles. Post-mortem findings are as follows:

Liver: Enlarged, hemorrhagic necrosis suggestive of acute congestion, capsular areas resembling anemic infarcts, gall bladder intact.

Testicles: Scrotal sac hemorrhagic with the testicles appearing normal with the exception of some induration.

Spleen: Spleen appeared essentially normal with exception of some congestion (blood).

Heart: Was extremely soft and hemorrhagic. Capsular areas resembling infarcts, and fibrotic in appearance.

Lung: Pulmonary infarcts with necrosis, foci of consolidation appearing as pale and gray patches, areas of hemorrhage and granulomatous-like lesions.

Kidneys: Appeared essentially normal with the exception of hemorrhage. Just prior to sacrificing the animal, blood was drawn for red and white blood cell counts. The red blood cell count was 3.45 million/c.mm, a decrease of 1.55 million/c.mm, calculated from the data obtained during the pre-infection base line studies. The white blood cell count was 22.95 thousand/c.mm, as compared to the animals normal count of 10.5 thousand/c.mm.

Microscopic examination: The following microscopic findings were present in all animals which expired (H and E staining procedure):

Liver: There is a considerable loss of cellular detail and diffuse granular change in the cytoplasm. Gross picture that of hemorrhage. The general architecture of the liver tissue appears well preserved. The central veins appear mildly dilated, areas of portal triads are intact with some being dispersed. Many occlused, engorged blood vessels, responsible for decreasing bile duct function and venous return. Many masses of fibrinoid with patches of increased fibroblastic cellular material. Hyperplastic but the hyperplasia is not that of white blood cell infiltration. Beginning of necrotic foci with a few areas consisting of inflammatory cells but not yet necrotic. Spotty areas of fibrinoid necrosis. No giant cells observed, indicating a young tissue insult. From the absence of a distinct white blood cell infiltrate, no defense mechanism is present.

Lung: Gross picture is that of hemorrhage, but not within the alveolar spaces themselves. The general architecture of the lung tissue is completely derranged. Bronchi appear normal without any demonstrable hyperplasia. There is a definite engorgment of all arteries and veins. No distinguishable respiratory lobule system is existant. Hemorrhage is confined to the parenchymal spaces. There are some areas of increased PMN infiltration.

Spleen: Gross picture is that of extreme hemorrhage without a white blood cell infiltration. The entire splenic architecture is non-intact. Extensive laydown of fibrinoid material is present. Germinal centers have been completely destroyed. No hyperplasia observed within the capsular tissue. Extensive congestion of the red pulp.

Heart: The myocardium is essentially unremarkable. Some fibrinoid material is seen. Microscopic results not coherent with gross findings. However, in an acute heart failure, one might expect such.

Testicular Tissue: There is an overall picture of autolysis with a resulting structure of degeneration, such changes being uniform. There is a gross hyperplasia of tubular cells, with a few giant cells observed within the tubules. Adipose tissue appears normal but containing some occlused, blood vessels. No hyperplasia of arteries but a distinct hyperplasia involving large mononuclear cells is seen throughout. Fibrinoid material present. Signs of degeneration without a distinct inflammation but an extreme monocytic infiltration is observed within the tubules.

Kidney: Signs of hemorrhage within the glomeruli without hyperplasia. Tubules intact with occlused arteries. No degeneration and the absence of an inflammatory reaction is observed.

Cortex..... Some hyperplasia of glomerular tufts are observed. Gross hemorrhage. Architecture intact but glomeruli are dearranged (pressure effect, result of).

Medulla..... Arteries are not engorged, no hyperplasia and no hemorrhage. Proximal to cortex shows some tubular hemorrhage without an inflammatory cell infiltrate.

The cause of death was most likely due to right-heart failure. Microscopic findings suggest a pre-requisite of toxemia, anemia and shock criteria. All findings agree with the acute regression observed in all animals. The hemorrhagic facet and mechanism is highly suggestive of "toxemic shock." The results of the Giemsa stains done on the above tissues are obscure. Small morphologic forms resembling B. bacilliformis were seen in numerous tissue sections. However, to call these forms B. bacilliformis, would be presumptive on our part at the present time. Cultures made from autopsy material are termed "negative for Bartonella" to date.

Rabbits No. 607 and 608:

The animals were immunized as follows, employing sonically-disrupted cells (B. bacilliformis - Strain 12):

Day	hedule
1	0.25 ml I.V.
6	0.50 " "
10	1.0 " "
13	1.5 " "
21	1.5 " "
25	Bled for titers

Both animals demonstrated a high precipitin titer on the 25th day. The animals were then left unattended until 80 days later, when the following injections were given employing viable organisms of Strain 12:

Rabbit No.	Day	Schedule
608	1	2.0 ml sub. cu. ("Booster Injection")
607	1	I.D. (x4), 0.3 ml/site
608	7	I.D. (.6), 0.3 ml/site

On the 16th day, both animals demonstrated epidermal lesions at the site of inoculation. However, the lesions presented by R-608 were more severe, larger, 20x28 mm and intensily inflammed and edematous. The lesions presented by R-607 disappeared without necrosis within 20 days while those of R-608 underwent necrosis and were still evident at the end of 35 days. Evidence for a more severe skin reaction in the immune animal is thus substantiated.

Rabbit No. 601

The animal was immunized as follows, employing viable organisms, the antigen labeled as "Somatic Antigen I (Strain 12):

Day	Route
1	0.1 ml I.V.
3	0.25 ml "
6	0.50 ml "
8	0.75 ml "
10	1.0 ml "
13	1.5 ml "
21	1.5 ml "

On the 96th day, the animal was given a booster injection of 2.0 ml viable organisms subcutaneously. Following a period of 6 days to allow for an anamnestic response, the animal was challenged.

Day (Post-booster injection)	Route
6	0.3 ml viable organisms into each testicle + 0.5 ml I.V.
8	0.4 " " " " " " " " " "
12	2.0 " " " " " " " " " "

The animal progressed as normal without any gross signs of infection or toxemia.

Rabbit No. 681

A series of preinfection hematological base line studies revealed animal to be in good health. The weight of the animal prior to injections was 5.5 lbs. The animal was injected as follows:

Day	Route
1	0.5 ml viable organisms I.V.
3	1.0 " " " "
7	2.0 " " " "

The animal showed no signs of degression during the injection schedule but died on the 8th day. Gross findings were those of hemorrhage. Upon autopsy, the peritoneal cavity was found to be filled with a milky-yellow fluid containing blood. All organs appeared normal. Cultures prepared from the peritoneal fluid and organs revealed no microorganisms. Microscopic, histological findings are incomplete on this date.

Rabbit No. 159

A series of preinfection hematological base line studies revealed animal to be in good health. The weight of the animal at this time was 12 lbs. The animal was inoculated as follows:

Day	Route
1	0.3 ml viable organisms into each testicle + 0.5 ml I.V.
3	0.4 " " " " " " " 1.0 " "
7	2.0 " " " I.V.

An orchitis present in both testicles was evident on the 10th day. The animal was irritable, apathetic and a weight loss of 3 lbs. was noted at this time. The animal degressed in health and died on the 14th day. External, gross findings were those of hemorrhage from the nostrils and a dark skin suggesting internal hemorrhage. In addition, both testicles were enlarged and hemorrhagic. Post-mortem findings were those of the previously described animals, highly suggestive of "toxemic shock." Cultures made from autopsy material were found to be contaminated after 5 days incubation. Two young, male rabbits (R-689 and R-613) were included as control animals, inoculated in the same manner as R-159. The only difference in the animals was weight and age; R-689 and R-613 weighing only 4 pounds with age comparable to the weights given. At no time did these animals present a picture of degression.

Rabbit No. 687

The animal was injected as follows employing the subcutaneous route of inoculation into the ears:

Left ear: 0.5 ml viable organism, Strain 12, cultivated in the O-L Medium

Right ear: 0.5 ml viable organism, Strain 12 cultivated in the semi-solid Medium

Lesions in both ears began to develop on the 10th day, post-inoculation, without inflammation. These cutaneous lesions reached a maximum on the 18th day, 12 x 10 x 6 mm in size. On the 30th day, both lesions had disappeared without undergoing necrosis.

The results of this particular pathogenicity study was interesting in view of the gross and histologic findings suggesting a prerequisite of toxemia, anemia and shock criteria. Such findings led us to investigate the role of bacterial metabolites in the pathogenesis of disease. In particular, the contributions of the Bartonella cell itself to the pathogenesis of the clinical features of Bartonellosis. Such findings are presented in the Immuno-Pathogenicity studies, to be described later.

I. Pathogenicity Study IX

In view of the findings obtained in Pathogenicity Study VIII, a study was initiated utilizing the same strain and the *Rhesus* monkey (splenectomized and non-splenetomized) as test animal. The comparative data of each individual animal and inoculation schedule is presented in Tables XXXIII through XXXXI.

It may be seen that no significant hematological changes were obtained during this particular study period. The animals did not show any outward signs of infection with the exception of MX-5 which developed small (3x5 mm) epidermal lesions at the sites of the intra-dermal inoculations. These

TABLE XXXIII

Animal No. MX-3
Animal Type: Monkey (splenectomized)

Day	RBC	WBC	Differential S	I	E	B	M	Comments
1		No Blood Obtained						
8	4.83	20.25	-	-	-	-	-	Bacteremia present
15		4.0 ml viable organisms sub. cu. (neck area)						
25		4.0 ml viable organisms I.P.						
29	5.33	17.15	62	33	0	1	4	
29		6.0 ml viable organisms sub. cu. (neck area)						
40	5.25	9.10	35	63	0	0	2	
47	5.75	7.50	55	42	0	0	3	
47		4.0 ml viable orgnisms sub. cu. (axillary area)						
60	5.55	8.20	53	45	0	0	2	

TABLE XXXIV

Animal Type: Monkey (non-splenectomized)
Animal No: MX-20

Day	RBC	WBC	Differential S	L	E	B	M
1		No blood obtained					
8	5.48	10.90	39	54	1	0	6
15		4.0 ml viable organisms sub. cu. (neck area)					
25		4.0 ml viable organisms I.P.					
29	4.98	11.40	40	58	0	0	2
29		6.0 ml viable organisms sub. cu. (neck area)					
40	5.50	7.45	60	40	0	0	0
47	4.62	16.00	53	46	0	0	1
47		4.0 ml viable organisms sub. cu. (axillary area)					
60	5.10	10.2	50	45	0	1	4

TABLE XXXV

Animal Type: Monkey (splenectomized)
Animal No. MX-4

Day	RBC	WBC	Differential S	L	E	B	M
1		No blood obtained					
8	5.47	8.60	44	53	0	0	3
15		2.0 ml viable organisms sub. cu. (neck area)					
25		2.5 ml viable organisms I.V.					
29	5.65	8.80	56	41	0	0	3
29		6.0 ml viable organisms sub. cu. (neck area)					
40	5.50	5.90	17	79	0	0	2
47	5.25	5.50	30	69	0	0	1
47		4.0 ml viable organisms I.P.					
60	5.35	6.75	40	52	1	2	5

TABLE XXXVI

Animal Type: Monkey (non-splenectomized)
Animal No: MX-20

Day	RBC	WBC	Differential S	L	E	B	M
1		No blood obtained					
8	5.48	10.90	39	54	1	0	6
15		4.0 ml viable organisms sub. cu. (neck area)					
25		4.0 ml viable organisms I.P.					
29	4.98	11.40	40	58	0	0	2
29		6.0 ml viable organisms sub. cu. (neck area)					
40	5.50	7.45	60	40	0	0	0
47	4.62	16.00	53	46	0	0	1
47		4.0 ml viable organisms sub. cu. (axillary area)					
60	5.10	10.2	50	45	0	1	4

TABLE XXXVII

Animal Type: Monkey (splenectomized)
Animal No: MX-4

Day	RBC	WBC	Differential S	L	E	B	M
1		No blood obtained					
8	5.47	8.60	44	53	0	0	3
15		2.0 ml viable organisms sub. cu. (neck area)					
25		2.5 ml viable organisms I.V.					
29	5.65	8.80	56	41	0	0	3
29		6.0 ml viable organisms sub. cu. (neck area)					
40	5.50	5.90	17	79	0	0	2
47	5.25	5.50	30	69	0	0	1
47		4.0 ml viable organisms I.P.					
60	5.35	6.75	40	52	1	2	5

TABLE XXXVIII

Animal Type: Monkey (splenectomized)
Animal No: MX-5

Day	RBC	WBC	Differential S	L	E	B	M
1	7.23	10.55	26	68	0	0	6
8	5.24	11.85	83	16	0	0	1
15		0.1 ml sub. orbital area (x3) 2nd 2.0 ml sub. cu. (neck area)					
25		4.0 ml sub. cu. (neck area)					
29	4.96	7.40	51	46	0	1	2
29		6.0 ml viable organisms sub. cu. (neck area)					
40	5.99	6.45	37	59	0	0	4
47	5.55	9.30	74	27	0	0	1
47		Animal received no injection					
60	5.30	10.00	38	58	1	1	2

TABLE XXXIX

Animal Type: Monkey (non-s;enectomized)
Animal No: MX-19

Day	RBC	WBC	Differential S	L	E	B	M
1	5.32	12.30	42	53	0	0	5
8	5.47	12.25	34	62	0	0	3
15		0.1 ml viable organisms sub. orbital (x3) 2nd 2.0 ml sub. cu.					
25		4.0 ml viable organisms sub. cu.					
29	5.87	9.45	69	28	0	0	3
29		6.0 ml viable organisms sub. cu. (neck area)					
40	4.38	14.50	58	41	0	0	1
47	5.32	11.70	37	59	0	0	4
47		e.0 ml viable organisms I.M. (front leg)					
60	5.43	12.0	45	50	0	0	5

TABLE XXXX

Animal Type: Monkey (non-splenectomized)
Animal No: MX-1

Day	RBC	WBC	Differential S	L	E	B	M
1	6.08	7.15	45	48	0	0	7
8	6.53	7.00	60	39	0	0	1
15		[illegible] ml viable organisms sub. cu. (neck area)					
25		2.5 ml viable organisms I.V.					
29	5.13	9.0	57	41	0	1	1
29		6.0 ml viable organisms sub. cu. (neck area)					
40	6.10	6.80	46	53	0	0	1
47	6.30	7.45	52	47	0	0	1
60	6.00	7.30	50	48	0	0	2

TABLE XXXXI

Animal Type: Monkey (non-splenectomized)
Animal No: MX-2

Day	RBC	WBC	S	L	E	B	M	Comments
1	5.20	20.75	-	-	-	-	-	Bacteremia
8	4.54	12.35	65	34	0	0	1	
15		2.0 ml serum sub. cu. (neck area)						
25		3.0 ml serum I.P.						
29	5.13	24.00	76	24	0	0	0	
29		No injection						
40	5.70	10.00	53	45	0	2	0	
47	6.98	7.10	49	50	0	0	1	
47		4.0 ml viable organisms I.P.						
60	5.35	10.5	47	53	0	0	0	

lesions remained as small, non-progressive, localized cutaneous lesions until the 45th day when they were no longer evident. In addition, all blood cultures, which were obtained on each occasion of hematological studies, were negative for B. bacilliformis. Blood films stained for the organism (Giemsa stain) were negative. The animals remained grossly normal and no complications were evidenced.

J. Pathogenicity Study X

This particular study employed the suckling mouse as test animal. Utilizing various routes of inoculation, i.e., intracranial, intramuscular and intraperitoneal, no demonstrable infection was observed. Blood cultures, cultures of tissues taken from mice which were sacrificed at various intervals and staining of such were negative for B. bacilliformis. Such observations were in accord with those of Perez-Alva, who observed that adult mice were resistant to the pathogenic effects of B. bacilliformis, even when substances known to reduce resistance were employed (78).

K. Pathogenicity Study XI

A small number of hamsters and guinea pigs were inoculated with large doses of B. bacilliformis, and various routes of inoculation were employed, i.e., intratesticular, intraperitoneal and intramuscular. In no instance was an infectious process demonstrated on the basis of gross examination, blood cultures and cultures and stains of various tissues. In addition, no epidermal lesions were observed following intradermal inoculations of viable or sonicated cell suspensions. From the results obtained in this and the previous study, it was concluded that the guinea pig, hamster and mouse possess a unique resistance to B. bacilliformis.

L. Pathogenicity Study XII

Previous studies on the biological properties of antiserum prepared against spleen and bone marrow, known as reticula endothelial immune serum (REIS), have been reviewed by Pomerat (86). A single intraperitoneal injection of 1.0 ml of anti-rat REIS with a C-F titer of 1:1600 has been shown to release a latent infection of Haemobartonella in carrier rats (8).

In view of our findings that splenectomy does not render the rabbit or monkey more susceptible to B. bacilliformis, it was concluded that such a defense mechanism may only play an insignificant role in the protection displayed by these animals. Therefore, a preliminary investigation was undertaken in our laboratory in order to determine the effect of rabbit, anti-guinea pig REIS on the guinea pig inoculated with B. bacilliformis.

The guinea pig REIS (inactivated) was first of all tested for hemolytic properties. When added in a dilution of 1:2 to washed guinea pig erythrocytes, no hemolysis was observed. Agglutination of such erythrocytes was observed at 1:256 but not at 1:512.

Various doses of the pooled REIS were injected intraperitoneally into guinea pigs; 1.0, 0.75, 0.5, 0.25, 0.20 and 0.15. Changes in erythrocyte count were drastic with the larger amounts of the REIS; the majority of the animals dying within 3 days post-injection with a severe hemolytic anemia. The ideal dosage in the given lot of guinea pigs employed in this study was established at 0.20 ml. Reduction of the red blood cell count in the animals receiving 0.20 ml REIS I.P. (0.5-1.0 million/c.mm.) was regularly observed within 48 hrs. after the administration of the serum. At this time the animals were inoculated with viable organisms of B. Bacilliformis. Six days later the reduction in the rbc count was still observed with the appearance of Bartonella-like inclusions adjacent to the red blood cells. Four days later the rbc count was back to its original volume and the Bartonella-like inclusions were no longer demonstrable. No significant decrease in the rbc volume nor any inclusion-like bodies adjacent to the red blood cells were observed in the following, enumerated controls:

(1) Animals treated with REIS; not challenged
(2) Animals treated with normal rabbit serum and then challenged with B. bacilliformis
(3) Animals challenged with B. bacilliformis; no treatment
(4) Animals treated with normal rabbit serum; not challenged

V. IMMUNO-PATHOGENICITY STUDIES

During Pathogenicity Study VIII, it became apparent that rabbits injected with viable cells of B. bacilliformis were highly reactive to subsequent injections of the homologous organism. The host reactions were those of anaphylactic shock with death occurring in the majority of such animals. Therefore, studies were initiated in direct concern to these findings in relation to the pathogenesis of Bartonellosis.

A preliminary experiment explored the susceptibility of rabbits to the lethality of a challenge dose of Bartonella bacilliformis after they has been infected with various strains of the intact organism. The established, "experimental infection" was well tolerated, with the animals remaining healthy and gaining weight readily during the experimental process. Nevertheless, as shown in Table XXXXII, when these infected animals were challenged with Bartonella bacilliformis (viable or disrupted organisms) 5 weeks after the onset of the infection, they did not survive the dose, which was not lethal for uninfected animals. Death displayed by these animals showed an association with endotoxic shock and an anaphylactic or hypersensitive response. This reaction was frequently severe enough to lead to the death of the animal within 48 hours.

Injection of sera, from animals showing both an increased susceptibility and a refractive state to the challenge dose, into normal, uninfected animals displayed the ability to produce a passive hypersensitive state in the latter animals. The results are shown in Table XXXXIII.

TABLE XXXXII

Mort lity in Rabbits following challenge
2 weeks after exptl. infection[1]

Animal No.	Strain No.	Challenge Material[2]	Results[3]
926	500	Viable Organisms	Death
927	500	Disrupted Organisms[4]	Death
928	14	Viable Organisms	Survival
936	14	Disrupted Organisms	Death
930	900	Viable Organisms	Death
931	900	Disrupted Organisms	Death
932	400	Viable Organisms	Death
933	400	Disrupted Organisms	Survival
934	700	Viable Organisms	Survival
935	700	Disrupted Organism	Death
690	15	" "	Survival
33	600	" "	"
693	16	" "	"
126	300	" "	"
692	900	" "	Death
34	14	" "	"
691	12	" "	Survival
644	400	" "	Death
623	500	" "	"
643	700	" "	"
645	10	" "	Survival
Animals in which <u>no</u> experimental infection was induced			
641	500	Disrupted Organism	Survival
642	700	" "	"
929	14	" "	"
925	900	" "	"
924	400	" "	"

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[1] Animals were infected through 3 weekly injections with viable organisms of the strain under question.

[2] 1.0 ml I.V. and 2.5 ml I.P.

[3] Mortalities were recorded for a 72 hr. period post-challenge.

[4] Sonically disrupted cell suspension.

TABLE XXXXIII

Response of Passively Presensitized Rabbits to Challenge Doses of Scnically Disrupted Bartonella bacilliformis

Sensitizing Serum	Amt. Serum Injected I.P.	Challenging Dose I.V.[1]	Results
R-926	1.0 ml	1.0 ml	No death
R-926	1.5 ml	1.5 ml	Death
R-934	1.0 ml	1.0 ml	No death
R-934	1.5 ml	1.5 ml	No death
None	None	1.5 ml	No death

[1]Disrupted cells of Strain 500 were administered 5 hrs. post-sensitization

Such data shows the amount of serum from a susceptible animal sufficient to cause increased response rather than protection when challenged under the given conditions. Serum from a refractive animal (R-934 did not induce such a state.

In this particular type of study, the results indicated that 5 strains of Bartonella bacilli' rmis were apparently more virulent than the others, i.e., 500, 400, 14, 900 and 700. In addition, the challenge dose could either be a viable suspension of the organism or a sonically disrupted suspension, results being comparable.

Comparative hematological, bacteriological and serological studies were carried out on these animals during the "experimental infection." These are summarized as follows:

Hematological. In the majority of animals studied, no significant changes in the blood cell picture were observed. However, 5 animals demonstrated a definite decrease in the red blood cell count. These results are shown in Table III.

TABLE XXXXIV

		RBC (million/c.mm.)		
Strain No.	Animal No.	Normal	Prior to Death	Amt. Decrease
500	R-926	4.60	3.61	1.00
900	R-931	4.73	3.00	1.73
400	R-933	5.53	3.68	1.85
14	R-936	5.84	3.10	2.74
700	R-935	5.00	3.60	1.40

Serological. Precipitin reactions were observed in immunodiffusion analyses when sera of the infected animals, taken prior to injection of the challenge dose, were analyzed opposite homologous suspensions of sonically disrupted cells of B. bacilliformis. In the majority of instances 1-2 precipitin bands were observed.

Bacteriological. All rabbit blood cultures taken during the infection were negative for Bartonella bacilliformis. In addition, all cultures taken from tissues at autopsy were negative.

From the results obtained during this study, it became more apparent that rabbits immunized with B. bacilliformis possess a unique sensitivity to subsequent injections of the homologous organism. In addition, serum of such animals could passively sensitize normal rabbits to metabolites of B. bacilliformis.

Employing predominantly Strain 500, the above studies were extended by determining the susceptibility in rabbits which had been injected with only 1 or 2 weekly doses of viable cells of B. bacilliformis. The resultant observations are presented in Table XXXXV. From this table it can be seen that animals injected with only 1 or 2 weekly doses are rendered nearly as susceptible as those animals receiving 3 weekly doses. In addition, such an increased state of susceptibility was demonstrated as early as 4 days following a single preparatory injection.

TABLE XXXXV

MORTALITY IN INFECTED[1] RABBITS FOLLOWING I.V. CHALLENGE[2]
3-7 DAYS FOLLOWING THE FINAL SUBCUTANEOUS INJECTION

NUMBER OF ANIMALS	NUMBER OF WEEKLY INJECTIONS	INTERVAL BETWEEN INFECTION AND CHALLENGE	PER CENT MORTALITY
4	1	4 days	50
4	1	7 days	50
4	2	3 days	25
4	2	4 days	50

1 Subcutaneous injection(s) with viable cells
2 Single intravenous injection with sonically disrupted cells

We next determined the susceptibility of groups of immunized rabbits to challenge doses of sonically disrupted organisms, over a longer period of time. Rabbits were injected subcutaneously with 3, weekly injections of viable organisms and then challenged at intervals of 1-12 weeks. The results of this study are presented in Table XXXXVI.

TABLE XXXXVI

MORTALITY IN RABBITS FOLLOWING I.V. CHALLENGE WITH SONICALLY DISRUPTED CELLS OF BARTONELLA BACILLIFORMIS AT VARIOUS INTERVALS AFTER INFECTION[1]

INTERVAL AFTER INFECTION	PER CENT MORTALITY
4 days	50
8 days	50
2 weeks	90
3 weeks	50
4 weeks	25
6 weeks	0
12 weeks	100

1 Three weekly subcutaneous injections of viable organisms

There is noted an increasing rate of mortality to the challenge injection from 4 days to 2 weeks. In addition, such a hyperreactive state could be demonstrated in only a few animals at 6 weeks while a high per centage of mortality was again observed at 12 weeks.

From such definitive data, it became apparent that the process of immunization might well induce a state of sensitization which was directly related to the optimal state of demonstrable hyperreactivity. Therefore, animals were infected with only 2 weekly subcutaneous injections of the viable organism and challenged at various intervals. As shown in Table XXXXVII, with less sensitization, the "primary" state of hyperreactivity was of a shorter duration and the "secondary" state of increased susceptibility was made manifest at an earlier time.

TABLE XXXXVII

MORTALITY IN INFECTED[1] RABBITS FOLLOWING I.V. CHALLENGE WITH SONICALLY DISRUPTED CELLS OF BARTONELLA BACILLIFORMIS AT VARIOUS INTERVALS

INTERVAL AFTER INFECTION	PER CENT MORTALITY
3 days	33
7 days	50
14 days	100
21 days	0
40 days	100

1 Two weekly subcutaneous injections of viable organisms

To substantiate our observation, animals were sensitized subcutaneously rather than infected with sonically disrupted cells and a lipopolysaccharide preparation of B. bacilliformis. Such animals were challenged intravenously with the LPS preparation and sonically disrupted cells respectively. The results of this study are presented in Table XXXXVIII.

TABLE XXXXVIII

MORTALITY AND SHOCK IN SENSITIZED RABBITS FOLLOWING I.V. CHALLENGE

SENSITIZING MATERIAL[1]	CHALLENGE MATERIAL[2]	INTERVAL BETWEEN FINAL SENSITIZING DOSE AND CHALLENGE DOSE (DAYS)	RESULTS
Endotoxin	S D Cells[3]	4	50% Mortality
Endotoxin	S D Cells	7	Shock
Endotoxin	S D Cells	14	No reaction
Endotoxin	Endotoxin	10	No reaction
S D Cells	Endotoxin	3	No reaction
S D Cells	Endotoxin	7	No reaction
S D Cells	Endotoxin	10	No reaction
S D Cells	Endotoxin	14	No reaction
S D Cells	S D Cells	10	100% mortality

1 Two weekly subcutaneous injections of the material under question
2 Single intravenous injection
3 Sonically disrupted cells

It was thus demonstrated that endotoxin could induce in such animals the increased state of hyperreactivity. However, the altered state was of a short duration. In addition, it was observed that when employing endotoxin as the challenging material, no state of hyperreactivity could be demonstrated.

Such an increasing state of hyperreactivity with time and death of the animals resembling anaphylaxis, led us to investigate the possibilities of an operative, immunological phenomenon. In the studies under question, it was observed that there is an early antibody response to the single or multiple (3) injection(s) of viable cells when analyzing the sera against sonically disrupted cells of B. bacilliformis. Table XXXXIX.

TABLE XXXXIX

IMMUNOLOGICAL STUDIES ON ANIMALS SURVIVING AND SUCCUMBING TO THE CHALLENGE DOSE OF SONICALLY DISRUPTED CELLS

INTERVAL BETWEEN SENSITIZATION AND CHALLENGE DOSES (DAYS)	NUMBER OF SUBCU. SENSITIZING INJECTIONS	RESULTS	NUMBER OF PRECIPITIN BANDS
5	2	Survival	1
5	2	Death	2
5	3	Death	2
5	3	Survival	1
9	3	Death	1
9	3	Survival	1
9	3	Death	2
9	3	Death	2
14	3	Survival	1
14	3	Survival	1
14	3	Death	2

Injection of sera, from animals demonstrating a hyper-reactive state, into normal, uninfected animals, displayed the ability to produce a passive hyperreactive (hypersensitive) state. The results of this study are shown in Table L.

TABLE L

RESPONSES OF PASSIVELY SENSITIZED RABBITS TO CHALLENGE DOSES OF SONICALLY DISRUPTED CELLS OF BARTONELLA BACILLIFORMIS

SENSITIZING SERUM[1]	AMOUNT SERUM INJECTED I.P.	CHALLENGING DOSE I.V.[2]	PER CENT MORTALITY
2	1.0	1.0	0
2	1.5	1.5	100
1	1.0	1.0	0
1	1.5	1.5	0
0	5.0	2.5	0
2	3.0	2.5	100
0	3.0	2.5	0
2	2.0	2.5	50
2 (young animals)	1.0	2.5	0
2	1.0	2.5	50

1 Two animals were passively sensitized per serum

2 Sonically disrupted cells were administered 5-8 hours post-sensitization

3 Refers to number of bands a particular serum gave via immuno-fiffusion analyses

Such data shows the amount of serum from hyperreactive animals (demonstrating 2 bands of precipitation) sufficient to cause increased response rather than protection when challenged under the given conditions. Sera from refractive or tolerant animals (demonstrating 0-1 band of precipitation) did not induce such a state. In addition, young animals could not be passively sensitized with serum of a hyperreactive animal as demonstrated with 2 young animals.

These particular studies employed passively transferring sera which had been taken from the infected or sensitized animals just prior to challenge. In a separate study, blood was withdrawn from two animals during the shock state just prior to death. Such sera, when administered intravenously in doses of 2.0-6.0 ml, into normal, control animals, had no adverse effects on the animal. Even though more conclusive studies are required, it was assumed that serum of an animal undergoing shock is devoid of circulating antigen-antibody complexes or antibody; the complexes and antibody being cell associated during this time. This conclusion was further based on the results of immuno-diffusion analyses when sera taken prior to challenge demonstrated 2 bands of precipitation while no bands were observed with serum of the same animal obtained during the shock state prior to death.

As previously stated, the most prominent aspect of these studies was that the death displayed by these animals showed an association with an endotoxic state of shock and also an anaphylactic response. Thus, when considering the results of the immunological studies and noting that hyperreactivity is delayed, followed by a state of tolerance which in turn is followed by a "secondary" state of maximal hyperreactivity, an analogy was apparent to an anti-anaphylactic state. To demonstrate our analogy, titers were determined on the sera of a group of animals demonstrating the hyperreactive and tolerant states during a 40 week period following immunization. It was observed that (1) the appearance of a hyperreactive state parallels the appearance of detectable antibody, (2) the state of tolerance is during that period when antibody is detected in its greatest concentration and (3) the "secondary" state of hyperreactivity is only demonstrable when the antibody titer falls to optimal concentration as determined during the primary state of hyperreactivity.

Following the death of the challenged animals, routine post-mortem examinations were preformed and tissue sections were processed and stained for histological studies. The results are presented below in discussion form:

<u>Kidney</u> - The pathogonomonic lesions of the generalized Shwartzman phenomenon, renal bilateral cortical necrosis, was a consistent finding in all animals examined. In animals dying 48 to 72 hours following the challenge injection, the gross and histological findings were most prominent. The kidneys were enlarged, the capsule separated easily and the body of the organ had a bluish, cyanotic color. The surface showed some petechial hemorrhages and occasionally, areas of grayish-white necrosis were observed. The cut surface showed extensive hemorrhages in the cortical aspects. No distinct areas of infarction were observed even though thrombi were demonstrated in some of the larger renal blood vessels.

Microscopic examination presented a consistent picture. Pathological findings presented themselves mainly in the cortex with the exception of a hyperemia which inovlved both the cortical and medullar aspects. The majority of glomeruli were occluded by a dense, homogeneous eosinophilic material resembling fibrinoid. Arteries and arterioles were similarly occluded with fibrinoid. Active congestion was observed throughout the cortex with the striking picture of only a mild, inflammatory cell infiltration. Cloudy swelling of the renal tubules was observed with the proximal tubules being more involved presenting a picture of hydropic degeneration.

Spleen - Pathological changes in the spleen were observed in all animals examined. The organ was most generally found to be enlarged and dark purple to black in color. In only several instances were distinct areas of necrosis observed. Microscopically, the identity of the over-all architecture was absent, replaced by acute congestion and material resembling fibrinoid. Such changes were most predominant in the sinusoids, being totally obliterated. The accumulation of fibrinoid was found to be greatest in the tissues immediately adjacent to the follicles with the cells of the germinal center giving the appearance of breaking-up, indicative of a toxic reaction.

Liver - Gross examination of the liver did not reveal any pathological changes with the exception of petechial hemorrhages and major blood vessels being occlused with fresh blood. Microscopically, venous thrombi were present with an extensive, acute congestion composed of large numbers of erythrocyte clumps. An early hydropic degeneration of the hepatic cell was characteristic finding, the cell appearing edematous. The absence of inflammatory cells was a striking feature. There was detected an uneven staining quality indicating selectivity of vascular distribution.

Lungs - Grossly, the lungs displayed hemorrhages and distinct areas of grayish white necrosis. Microscopically, the tissue showed evidence of active congestion with dilatation of the blood vessels in the septal wall and thrombi and emboli were existent within the pulmonary arteries. The absence of an inflammatory cell infiltration was again a striking feature in view of the edematous appearance of the tissue.

Heart - No gross changes were observed in the heart with the exception of being enlarged in a number of cases and evidence of blood pooling in the right ventricle. Microscopically, the over-all architecture appeared rough, indicative of early anoxic changes. There was a distinct fading and loss of muscle fiber striations with the normal nuclear position being disturbed. There was evidence of a loss of myoplasm. In several instances, subendothelial accumulations of fibrinoid material were observed with fragmentation of muscle fibers about the blood vessels, primarily in the perivascular areas. In other instances, perivascular edema was observed causing a cuffing effect, with the heart muscles appearing as being split due to a loss of fluid(s). Strongly indicative of a toxemic shock was an arteriole phenomenon with dilatation and engorgement of arteries and subsequent loss of integrity. The condition was attributed to tissue hypoxia leading to an increased permeability of the endothelium and connective tissue, mainly due to hydrostatic changes.

<u>Thymus</u> - Gross examination of the thymus revealed extensive hemorrhage and thymic atrophy in excess of that which occurs normally in the rabbit. The over-all identity of architecture had been lost with the tissue appearing cracked and uneven suggestive of atrophy and loss of firmness. In several instances, extensive hemorrhage was present throughout the gland and occlused and congested blood vessels with red blood cells was a consistent finding. The interlobular septa of the connective tissue was not intact resulting in separated lobules. Material resembling fibrinoid was present throughout the organ.

DISCUSSION

Re-evaluation of Bartonella bacilliformis. During the early stages of our investigation, we encountered certain difficulties which warranted consideration. Several of these problems are enumerated below:

1. The appearance of Bartonella-like structures in uninoculated media which had been supplemented with bood products.

2. The observation of dark, Giemsa staining inclusions in and/or on red blood cells obtained from apparently normal laboratory animals.

3. The observation that the numerous isolates sent to us did not conform to the classical description of B. bacilliformis.

4. The conclusion that the literature in direct concern to Bartonellosis and its etiological agent was confusing and contradictory.

5. The frequent appearance of contaminating organisms which could quite possibly be confused with B. bacilliformis.

6. The inability to confirm some of the reported investigations, i.e., growth characteristics, growth in embryonating avian eggs and tissue cultures, experimental asymptomatic Bartonellosis and cutaneous lesion development in the Rhesus monkey.

Thus, in order for us to pursue our investigations from the standpoint of validity, the identity and characterization of Bartonellosis and B. bacilliformis had to be re-evaluated.

Since we had not encountered this organism previously and Bartonellosis is nonexistant in the United States, it appeared most feasible to work directly with Peruvian investigators who were studying certain aspects of Bartonellosis at that time. Thus, a period of time was spend in Lima, Peru, working directly with Dr. Aristides Herrer, Instituo Nacional de Salud, Lima, Peru and Drs. Manuel Cuadra, Pedro Larrear and Oscar Urteaga, Hospital Dos de Mayo, Lima, Peru. Our main aims were to learn: (1) of the disease Bartonellosis, (2) how B. bacilliformis is identified and cultivated, (3) how to obtain specimens from patients presenting either form of the disease and (4) how to transport several isolates back to our laboratories.

Described briefly below are certain aspects of our studies conducted in Lima, which emphasize the inevitability of our previously encountered problems.

Isolation techniques. When isolating the organism from a patient with the anemic form of the disease, two methods appear to be used most frequently. One employs inoculating a small portion of patient's blood, which has been drawn aseptically and allowed to clot, into Noguchi's semi-solid medium. The other method employs inoculating approximately 5 ml of patient's blood directly into a tryptose medium supplemented with beef extract and sodium citrate. The above techniques have been used with success. However, if the disease is complicated with another microorganism, i.e., Salmonella, the process of selection for B. bacilliformis is to no avail.

Cultivation (media). Four media are primarily used in the cultivation of B. bacilliformis. A semi-solid medium, blood agar, blood-broth and an agar-slant tissue culture medium employing mouse embryo tissues. All of these media require blood, serum or plasma, with the latter supplemented with hemoglobin. The morphology of the individual organism and colonies are most distinct in the semi-solid medium. The agar slant-tissue culture medium employing minced mouse embryo supports proliferative growth within 2-3 days. When subculturing from this media to the semi-solid, optimal growth is observed within 2 days. Blood agar slants are sometimes used, but due to the length of time required for the appearance of colonies, is unsatisfactory. The blood-broth is used primarily for the isolation of the organism from patients.

Morphology. In the semi-solid medium, the organisms occur in large and small, irregular dense collections measuring up to 25µ or more in diameter. Such colonies are easily observed grossly and when observed under dark field illumination, such colonies are seen to be composed of distinct, cocco-bacillary forms.

Identification. The procedures used in the identification of B. bacilliformis are far from satisfactory. Identification is made only from the results of the cultural and morphological criteria given above. No biochemical serological or physiological aspects are known concerning the organism. Therefore, a pattern of distinct characteristics ordinarily used in bacterial identification is unavailable.

Diagnosis of the Clinical Disease in Man. Diagnosis of the hematic syndrome (Oroya fever) is made primarily from examination of peripheral blood. There is always a degree of anemia which rapidly becomes severe. Marked degenerative activity is indicated in both the erythroid and myeloid series. The number of nucleated red cells may be exceedingly high and reticulocytes may increase to 75% in severe cases. Such hemopoetic activity is also apparent in bone marrow studies. The pathognomic sign of Oroya fever is the presence of B. bacilliformis in, or on, the erythrocyte. Up to 90% of the red cells may be parasitized in a heavy infection. The organisms may occurr singly, or be distributed in Y, V or chain appearance. In intense infections, the organisms may also be seen in the circulating monocytes. A rapid diagnosis can be made through information of the above manifestations.

Unlike Oroya fever, verruga peruana is characterized by a distinctive appearance and diagnosis is readily established upon physical examination. The verruga nodules vary considerably in appearance, but are basically of three types and may be distinguished according to evolution, shape, situation, size, disposition and other general aspects of the eruption. With the information obtained during our studies in Lima and successful transportation of numerous strains to our laboratory, a series of experiments were performed in order to re-evaluate the genus Bartonella. From the results of the biochemical, physiological, morphological and cultural studies, it was apparent that there were certain distinct characteristics of B. bacilliformis. The organism is typically small, cocco-bacillary in morphology and stains reddish-purple with Giemsa stain following methanol fixation. It does not stain with any of the common aniline dyes nor with the acid fast stain. It is motile, demonstrating a motility, visible by dark field illumination which is idicative of unipolar, lophitrichous flagella. Contrary to previous investigations (67), B. bacilliformis is micro-aerophilic, with the optimal hydrogen ion concentration being 7.8 and the optimal temperature for metabolism and reproduction being between 25° and 28° C. The most distinctive characteristic of the organism is its extreme fastidious nature, possessing a strict need for accessory growth factors which are present in blood and certain blood products. Such factors are not limited to the X and/or V fractions. Due to the absolute necessity of such factors, certain characteristics could not be obtained, i.e., action on nitrate, citrate utilization and carbohydrate fermentations.

The procedures employed in establishing the identity of B. bacilliformis are far from satisfactory. Howevere, a number of our findings enabled us to make a more concise identification of the organism and provided us with certain tools with which to define a taxonomic status for the organism. Through the techniques of immuno-diffusion, it was observed that B. bacilliformis has a complex antigenic character. The examined isolates share this antigenic complement and several isolates possess strain specific antigens. One of the antigens is a somatic, constituent which is biologically and/or antigenically similar, if not identical, with that of various heterologous Gram negative microorganisms. In addition to common somatic antigen, B. bacilliformis possesses a number of additional common antigens with H. parainfluenzae, Herellea vaginicola and S. marcesens.

The Fluorescent Antibody techniques (FA) employing conjugated Bartonella anti-serum, provided us with a highly specific test for the identification of B. bacilliformis. Heterologous, Gram negative organisms could not be stained with this conjugate. These results indicated that such conjugates could be useful in providing a rapid, specific test for the demonstration of B. bacilliformis in blood, bone marrow and skin biopsies.

It was also observed that B. bacilliformis possesses a hemagglutinin. Such a capacity provided a supplementary tool for identification purposes, standardization of cell suspensions and possibly for the diagnosis of Bartonellosis.

The reported review of Peters and Wigand (79) served as a reference when attempting to correlate our findings with those of others in direct concern to the taxonomy and nomenclature of B. bacilliformis. As presented above, and in view of previous findings, B. bacilliformis possesses characteristic and properties typical of bacteria in contrast to Haemobartonella, Eperythrozoon and the Rickettsiaceae. Namely, B. bacilliformis is cocco-bacillus, demonstrates retracted cytoplasm and cell walls upon electron microscopy, are motile due to unipolar flagella, reproduce by binary fission and can multiply on culture media devoid of viable tissue cells.

It thusly appears feasible to not only separate B. bacilliformis from the animal Bartonella but to place it among the true bacteria. Due to its particular growth requirements and distinguishing biological properties, it is suggested that the genus Bartonella, species Bartonella bacilliformis, be placed in the Brucellaceae.

Pathogenicity of Bartonella bacilliformis.

In relationship to the organism B. bacilliformis, little is known concerning its pathogenicity when using the direct approach of correlating (1) incidence, (2) resistance and (3) the infectious process, with the disease Bartonellosis. Experimentally, it has been established that the organism is only capable of causing an atypical demonstrable reaction in certain species of animals. From this, we are led to conclude that the host plays a definite role in regard to (1) non-susceptibility, (2) natural immunity and (3) non-specific resistance to infection.

Non-susceptibility is primarily illustrated by the characteristic occurrence of infection in certain species but not in others. Certain physiological factors such as body temperature and diet contribute to nonsuceptibility, but such factors are more apparent than real as the infectious process may be manifested only when such physiologic conditions are altered. In addition, the requirements by an organism for particular accessory growth factors may limit its ability to multiply within a given host.

Natural immunity is attributed to antibodies which are present or appear in man and laboratory animals without obvious external stimulus. The concentration of such is most generally low. The origin of these antibodies is by no means certain and evidence suggests that they are not, in some instances, specific for a given antigen(s) organism). Particularly since there is a heterogenous group(s) of organisms which cross-react with these antibody-like substances. The subject of natural antibodies has been extensively reviewed elsewhere (133 A).

Non-specific resistance manifests itself primarily in the physical and chemical barriers presented by epithelial tissue. As related to B. bacilliformis, there might be a possible significance of this type of resistance as the disease is normally induced by the bite of a sandfly. The importance and/or necessity of the bite in establishing infection and the resultant effect on the host tissue and other such implications are unknown.

There are other factors which lie outside the scope of Immunology but which indirectly determine the opportunity for infection, the development of immunity and host-response(s) of the established, disease process. These factors pertain to the general state of health or debilitation of the host, climate, geographical location and socio-economic conditions.

An acquired immunity to an infectious disease or, moreso, to a given organism(s) imports to the host an unabsolute immunity in that the immunity displayed is of variable degree and intensity against the same or other infectious agents; depending primarily upon the relationships of the causative organisms. In consideration of the lessened severity of the disease in the endemic zones, the usual lack of reinfection in a previously infected individual and the demonstration of agglutination of C-F titers in convalescing individuals, indicates that there is an acquired type of immunity in Bartonellosis.

Taking the preceding discussion into consideration, several factors concerning the parasite B. bacilliformis and the host(s) which pertained to our experimental appraoches and/or results obtained inexperimental pathogenicity sutdies are as follows:

The parasite, B. bacilliformis

1. The organism has a strict need for accessory growth factors which are present in blood and blood products and, most likely, in other host tissues, fluids and transudates. The specific metabolic requirements are unknown.

2. The optimal hydrogen ion concentration and optimal temperature for metabolism, growth and reproduction is pH 7.8 and 28 C respectively.

3. The degree and mode of invasive capability is not specifically known. However, several of our studies indicated that the intratesticular route of inoculation, where a depot of organisms is established, may be superior to other routes through which dissemination of the organism occurs.

4. The organism produces an endotoxin which is antigenically identical to the somatic endotoxic constituent of various Gram negative organisms. Such a metabolite provokes a more or less violent host tissue response, and it appeared that this fraction was responsible for the observed strain differences shown for rabbit virulence. However, the mechanism of toxicity does not simulate that of other organisms in that the macromolecule is of relatively low potency (toxicity).

5. The antigenic structure of B. bacilliformis is rather complex and demonstrates a phylogenetic relationship with other organisms. Its antigenic complement is comprised of agglutinogens, preciptinogens and hemagglutinin.

6. B. bacilliformis will multiply in vitro in the blood of many species but to varying degrees even among a given species.

7. The organism will induce repsonses in vivo in relatively few species with a variation within a given species. The host responses are mainly hematological and immunological, i.e., hemopoetic alterations and antibody production. In such animals, a distinct use in the peripheral white blood cell count was noted approximately 3-4 weeks following the initial injection of viable organisms. Accompanying such a hemopoetic change was an evident decrease in the peripheral red blood cells but not to such a critical extent as to be indicative of an anemia.

8. The organism is apparently transmitted by Phlebotomus verrucarum and P. noguchii which are restricted to given geographical areas of Peru, Columbia, and Ecuador. Such areas are those in which the disease occurs most frequently.

The host in relation to Bartonella bacilliformis

1. A number of animal species were found to be non-susceptible to spontaneous infection, i.e., adult mice, suckling mice, guinea pigs, hamsters and monkeys. The rabbit appeared to be the most susceptible species with splenectomized rabbits demonstrating no greater susceptibility than nonsplenectomized rabbits.

2. The majority of experimental animals employed have a normal body temperature of 37° C or above.

3. No normal antibodies to B. bacilliformis were demonstrated. The antibodies which are actively produced occur late in the course of immunization and are apparently non-protective in nature. However, such immunized animals demonstrated positive dermal reactions to I.D. injections of viable cells or disrupted cells whereas normal animals did not. In addition, the immune serum could not be employed for growth purposes of the organims; indicative of a neutralizing antibody.

Immuno-Pathogenicity of B. bacilliformis

Hyper-susceptibility. Certain investigations have emphasized the significant role of bacterial metabolites in the pathogenesis of disease; namely, the role of bacterial endotoxins in the pathogenesis of Brucellosis (104,105,106). For instance, it was found in man that those individuals previously infected with Brucellae were more susceptible to the toxic actions of Brucella endotoxin than control, uninfected subjects (2).

In experimental animals Boivin et al. (14,15) demonstrated that mice injected with Salmonella typhimurium possessed an increased sensitivity to E. coli endotoxin. Similarly, Bennet (12) has shown that rabbits infected with E. coli were more susceptible to the lethal effects of typhoid vaccine. More recently, Dubos et al. (32) have demonstrated an increased death rate following endotoxin injection into mice which had been infected with Staphylococcus and Mycobacterium. Pirsch et al (85) found that guinea pigs, infected with Coxiella burnetii, were hyperreactive to various endotoxins and succumbed much more rapidly than uninfected animals. Abernathy et al. (1) demonstrated an increased susceptibility to the lethality of Brucella, E. coli and typhoid endotoxin in mice infected with Brucella melitensis or Brucella abortus.

Such information was obtained in our laboratory with Bartonella bacilliformis; namely, infection* with Bartonella bacilliformis increased the susceptibility of rabbits to the lethality of Bartonella metabolites (endotoxin).

The mechanisms by which infection alters the reactivity to bacterial metabolites are not clear. It has been conjectured that during infection destruction of bacteria by host defense mechanisms liberated endotoxin in a sufficient quantity which then caused the development of resistance, much as that which occurs following injection(s) of endotoxin. The absence of resistance may reflect only that these infections provided an inadequate stimulus for its development. However, it is also possible that resistance develops but is overcome by those factors responsible for the increased susceptibility. Although it is conceivable that this susceptibility may result from a general reduction in the ability of the infected host to resist toxic agents of any nature, several observations serve to controvert such supposition. The degree of infection established by us with Bartonella bacilliformis and by other workers with Brucella (1), was never sufficient to give clear-cut clinical evidence of disease; all animals

*The term "infection" is used here to describe the altered state of the animal which has received doses of viable cells of Bartonella bacilliformis.

being active, gaining weight normally and appearing as healthy as uninfected control animals.

To explain the susceptibility to endotoxins during infection. Boivin and Delaunay (15) advanced the hypothesis that the adminstration of endotoxin, through an enhancing action upon infections, converted a dormant infection into a lethal one. In support of this interpretation, these workers observed that death, after endotoxin injection, occurred over a period of 20 days. This concept is supported by others (32) who concluded that the endotoxin converted a chronic bacterial infection into an acute process with the explosive multiplication of organisms. However, the studies by Abernathy et al. (1) did not reveal an increase in the number of Brucella cultured from the spleen after the administration of endotoxin. In addition, deaths after endotoxin in Brucella-infected mice uniformly occurred within 8-48 hr. It is more likely that these deaths represented a direct action of endotoxin in a sensitive animal rather than a secondary process. These results are in close accord with those of Pirsch et al. who failed to demonstrate in guinea pigs infected with C. burnettii any increase in rickettsia following the administration of endotoxin (85). These investigators suggested that the hyperreactivity of the infected guinea pigs to endotoxin was a manifestation of the generalized Shwartzman phenomenon.

The methods employed and results obtained during the course of this investigation indicated that sensitivity to B. bacilliformis metabolites offers an excellent experimental condition for studying some of the major effects of endotoxin, i.e., fever, shock, lethality and the generalized Shwartzman reaction. In addition, the above described studies directly employed an experimental infectious state with intact organisms or a sensitized state with sonically disrupted organisms. Employing these materials as inocula, a complement of antigens were exposed to the host rather than a single antigen such as the endotoxin macromolecule. Such conditions thusly simulated more closely the human model during an infectious process. Experimental procedures of this nature are of utmost concern. For instance, it is a well established fact that occlusive vascular lesions characteristic of the generalized Shwartzman phenomenon are encountered in a variety of human diseases, i.e., bacterial infections, polyarteritis nodosa, disseminated LE, thrombotic thrombocytopenic purpura and in obstetrical disorders (19,27,56). Therefore, carefully evaluated procedures directed towards an explanation of such host reponses are essential.

A direct immunological basis for explaining enhanced susceptibility or hyperreactivity of experimentally infected animals has not been substantiated (109,110,111,112). These authors primarily demonstrated that (1) the induction of the hyperreactive state was nondependent upon infection, (2) transfer of the hyperreactive state could not be accomplished by cells or serum and (3) shock appeared to be the only observable alteration of response to endotoxin in the hyperreactive state.

Our findings have demonstrated that the production of the hyperreactive state is dependent upon a sensitization (or infection), followed by a latent period of short duration. The state is then demonstrated upon an eliciting, challenge injection of the sensitizing material. Manifestations of shock, lethality and the generalized Shwartzman phenomenon were the observed host response to the challenge injection. A maximal altered state of the host descriptive as hyperreactivity, was demonstrated with a concurrently, detectable existence of circulating antibody at a sufficient, optimal titer. When such antibodies were found in too great a concentration, a refractive or tolerant state to the challenge material was demonstrated.

Significance of hyperreactivity. The mechanisms by which patients in the "critical stage" of Bartonellosis develop a characteristic symptomology of malaise, almost sudden onset of fever, chills, nocturnal sweating and frequently a remittent type of fever due to secondary infection has not been clearly elucidated. In particular, the contributions of the Bartonella cell itself to the pathogenesis of these clinical features have not been investigated.

The term "critical stage" of Bartonellosis has been applied to the period of transition in which the organism suddenly disappears from the red blood cell. Even though the mechanism of this change is controversial, it is a fact that within a matter of days, the organisms disappear from the peripheral blood. The hematologic sign of this transition are enumerated as follows: (1) A morphologic change in the form of the organism from a bacillary to a coccoid form occurs with the appearance of bizzare, pleomorphic forms. (2) Decrease in the number of parasitized red blood cells and in the number of organisms in the peripheral blood. (3) A rise in the erythrocyte count and hemoglobin. (4) A reduction in the indirect hyper-bilirubinemia to normal. (5) An increase in the number of reticulocytes indicative of hematic regeneration. (6) A decrease in the macrocytosis; even a tendency to microcytosis. (7) A lymhocytosis with a shift to the "right" of the polymorphonuclear series. With this transition, the clinical signs of fever and subicteric tinge of the skin and sclera disappear. In addition, due to the prompt rise in the number of erythrocytes, the symptoms of anemia, such as fainting, dizziness, tinnitus, etc., disappear, as well as the hemic heart murmurs. In essence, the patient appears to be convalescent.

However, this sequence of events does not always occur. There may be an increased severity of the clinical course, which is due to intercurrent infections in the majority of cases. In such instances, the symptomology begins with malaise and fever of moderate degree, becoming rapidly malignant, with death occurring usually on the seventh day, sometimes sooner but very seldom later than the tenth day after the onset. The clinical appearance of Salmonella complication differs from classical-typhoid fever by almost sudden onset of fever, rarity of the stuporous state, rarity of splenomegaly, frequent chills, hypertension, nocturnal sweating and frequently a remittent type of fever. In addition, Salmonella may be easily isolated from the patient's blood. Such symptoms indicate a toxemia and the effects suggest that the fore-running course of Bartonellosis influences the distinct pathogenesis of the Salmonella complication.

The exact contribution of the symptomatology of complicated Bartonellosis to Salmonella endotoxin is purely conjectural. However, the accentuation of susceptibility to Salmonella endotoxin in individuals who have had the active hematic form of Bartonellosis, suggests that this is a manifestation of generalized hyperreactivity (increased susceptibility) to the endotoxin. For further support of this concept, it has been found that the reactions of such subjects in the critical stage of Bartonellosis react in the same manner when the secondary infection involves other Gram negative organisms known to produce endotoxin, i.e., Salmonella paratyphi, E. coli, Salmonella enteritidis, Salmonella schotemulleri, Brucella melitensis, Salmonella typhimurium. Such data reflect the role of endotoxic shock since it has been known for some time that all bacterial endotoxins possess similar toxic actions for humans.

From these observations in humans, coupled without preliminary observations in the rabbit, the role of endotoxin in the pathogenesis of Bartonellosis may be envisioned as follows: Upon invasion of the host by Bartonella bacilliformis, the hematic syndrome of an anemia develops which terminates in a transition period ("critical stage") due to natural host defense mechanisms. However, this results in the disruption of the bacterial cells, with liberation of endotoxin as evidenced from the finding of bizzare spherical, granular organisms. If a sufficient quantity floods the tissues, the host responds with a febrile and systemic response. However, it appears that during the infectious and post-infectious process, a state of increased susceptibility develops which renders the host more sensitive to the actions of endotoxin from a secondary bacterial invader.

SUMMATION

1. A review of the literature was presented in direct concern of Bartonellosis and its etiological agent, Bartonella bacilliformis.

2. B. bacilliformis was found to be a cocco-bacillus, usually less than 1.0 micron in the greatest dimension. Giemsa stain following methanol fixation and dark field microscopy provided the best means for studying its morphology.

3. Various strains of B. bacilliformis were grown in solid, semi-solid, overlay, and agar- lant tissue culture media, when the pH was 7.6-7.8, temperature was 28° C, microaerophilic conditions were provided and when blood products were incorporated in the media.

4. Enrichments and supplements (Difco), hemin or dehydrated hemoglobin could not replace fresh blood products in media employed for the cultivation of B. bacilliformis.

5. Numerous strains of B. bacilliformis were susceptible to the effects of freezing, cold storage and lyophilization when employing various menstra as suspending media. The organism was best maintained in a semi-solid media at 28° C.

6. No growth or viability of the organism could be demonstrated in embryonating avian eggs or various tissue cultures under given conditions.

7. A fluorescent antibody technique was described for the specific identification of B. bacilliformis from culture and the organism was demonstrated to possess a hemagglutinin.

8. Applications of the tube agglutination procedure for the identification and standardization of B. bacilliformis were presented.

9. Utilizing the techniques of immunodiffusion, (a) homogenicty of the various strains of B. bacilliforms was determined, (b) the minimum number of antigens present in each strain were enumerated, (c) common antigens were demonstrated among B. bacilliformis and various heterologous Gram negative organisms, (d) the distribution of strain specific antigens was determined.

10. The rabbit was found to be more susceptible to B. bacilliformis than were the Rhesus monkey, guinea pig, hamster or suckling mouse. In the rabbit the infectious process could best be followed by peripheral blood studies. A combination of the intravenous and intratesticular routes resulted in a more profound experimental infection than when other routes were employed. Splenectomized rabbits did not appear more susceptible to infection than non-splenectomized rabbits.

11. Injection of rabbit, anti-guinea pig RES serum followed by injection of B. bacilliformis into guinea pigs produced a definite regenerative type of anemia. During the period of maximal red blood cell decrease, inclusions resembling B. bacilliformis were found adjacent to and/or in the red blood cells.

12. An endotoxic preparation of relatively low potency was extracted from _B. bacilliformis_.

13. _Bartonella_ sensitized rabbits were found to be more susceptible to the lethality of _Bartonella_ metabolite (endotoxin) than were normal rabbits. Such animals exhibited systemic reactions resembling anaphylaxis and endotoxic shock. The degree of host response (shock and/or manifestations of the generalized Shwartzman reaction) was dependent upon the level of antibodies specific for a somatic, endotoxic antigen.

LITERATURE REVIEW

1. Abernathy, R.S., G.M. Bradley and W.W. Spink. 1958. Increased susceptibility of mice with Brucellosis to bacterial endotoxins. J. Immunol. 81:271-275.

2. Abernathy, R.S. and W.W. Spink, 1958. Studies with Brucella endotoxin in humans: The significance of susceptibility to endotoxin in the pathogenesis of Brucellosis. J. Clin. Invest. 37:219-231.

3. Aldana, L. 1929. Bacteriologia de la enfermedad de Carrion. La Cron. Med., Lima 46:235-285.

4. Aldana, L. and T. Munoz. 1945. Penicillina y enfermedad de Carrion. Rev. Med. Peruana 18:343.

5. Aldana, G.L., R. Bastelumendi and J. Dieguez. 1948. Streptomycin in Carrion's Disease. Arch. peruanos pat. y. clin. 2:323-339.

6. Aldana, G. Luis Antonio. 1949. Contribution at estudio de la complicacion salmonellosica en la enfermedad de Carrion. Rev. san. policia 9:9.

7. Aleksandrowicz, J., J. Kowalczykowa and Z. Przybylkiewcz. 1954. Acute reticulosis in the course of a Bartonella infection. Przegll Lek., Krahow 10:347-351.

8. Anigstein, L. and C.M. Pomerat. 1945. Reticulo-endothelial immune serum (REIS): IV. Experimental production of anemia and Bartonellosis in rats by inhibitory dosage. Tex. Rep. Biol. Med. 3:4, 545-557.

9. Antonez, D. 1890. Enfermedad de Carrion. Cron. Med., Lima 7:290.

10. Barton, A. 1909. Descripcion de elementos endogobulares hallados en lose enfermos de fiebre verrucosa. Cron. Med., Lima 7:26.

11. Barnett, H.C. 1962. Sandflies and sandfly-borne diseases. Biological Transmission of Disease Agents. Academic Press, Inc. New York, pp. 83-91.

12. Bennett, I.L., Jr. 1948. Observations on the fever caused by bacterial pryogens. I. A study of the relationship between the fever caused by bacterial pyrogens and the fever accompanying acute infections. J. Exptl. Med. 88:267-278.

13. Biffi, U. and J. Gastiaburu, 1903. Sabre las hemoaglutininas de la sangre humana y la hematologia de la enfermedad de Carrion. Cron. Med., Lima 19:113.

14. Boivin, A. and A. Delaunay. 1942. Les antigenes gludico-lipidiques, facteurs favorisant les infections bacteriennes. Antigenes glucido-lipicliques et "agressines." Revue d'Immunol. (Paris) 7:19-207.

15. Boivin, A and A. Delaunay. 1943. L'action pro-infectieuse des antigenes glucido-lipidiques: Faits nouveaux et mecanisme de l'effect agressif. Revue d'immunol. (Paris) 8:148-160.

16. Breed, R.S., E.G.D. Murray, and A.P. Hitchens. 1948. Bergey's Manual of Determinative Bacteriology. Sixth Addition. The Williams and Wilkins Co., Baltimore.

17. Breed, R.S., E.G.D. Murray, and N.R. Smith. 1957. Berey's Manual of Determinative Bacteriology, Seventh Edition. The Williams and Wilkins Co., Baltimore.

18. Brumpt, E. and L.C. Brumpt. 1942. Bartonellosis: appearance in Colombia and epidemiologic study. Ann. de Parasitol. 19:1-50.

19. Brunson, J.G. and R.L. Davis. 1955. Systemic fibrinoid diseases. Similarity to experimental lesions in rabbits. A.M.A. Arch. Pathol. 60:593-609.

20. Brunson, J.G., L. Thomas and C.N. Gamble. 1955. Morphologic changes in rabbits following the intravenous administration of meningococcal toxin. II. Two appropriately spaced injections; the role of fibrinoid in the generalized Shwarrzman reaction. Am. J. Pathol. 31:655-667

21. Caballero, C.A. and F. Mayoral. 1942. Human bartonellosis in Chile. Rev. de Med. y. Cir., Barranquilla 9:20-38 and Bol. Med. Social., Santiago 9:700-715.

22. Camargo, L.P. 1939. Bartonellosis (verruccus fever) of Guaitara Valley in Colombia. Rev. Fac. de Med., Bogota 7:467-501.

23. Camargo, L.P., P. Cifuenutes and M.S. Herrera. 1940. First case in Bogota. Rev. Fac. de Med., Bogota 9:351-359 and Bol. Ofic. san. Panama 19:1070-1077.

24. Camargo, L. Patino. 1940. New focus of bartonellosis in Amerkica: epidemic in Colombia. Bull. Office Internat. d'Hyg. Pub. 32:570-571.

25. Cherry, W.B., M. Goldman and T.R. Carski. 1960. Fluorescent Antibody Techniques in the diagnosis of Communicable Diseases. Public Health Service Publication No. 729. U.S. Dept. of Health, Education and Welfare, U.S. Gov't. Printing Office, Washington, D.C.

26. Conn, H.J., J.W. Bartholomew and M.W. Jennison. 1956. Staining Methods, Leaflet IV, of the Manual of Methods for Pure Culture Study of Bacteria. Committee on Bacteriological Technic of the Society of American Bacteriologists. Biotech Publications, Geneva, New York.

27. Craig, J.M. and D. Gitlin. 1957. The nature of hyaline thrombi in thrombotic thrombocytopenic purpura. Am. J. Pathol. 33:251-

28. Cuadra, M. 1956. Salmonellosis complication in human bartonellosis. Tex. Rep. Biol. Med. 14:97-113.

29. Darling, S. T. 1911. Verruca peruana. J.A.M.A. 57:2071-2074.

30. De Vault, V. T. 1952. Treatment of bartonellosis with antibiotics. Ann. N.Y. Acad. Sci. 55:1222-1227.

31. Deutsch, H. F. 1952. Separation of antibody-active proteins from various animal sera by ethanol fractionation techniques. In: Methods in Medical Research 5:284-300.

32. Dubos, R. J., R. W. Schaedler and D. Bohme, 1957. Effects of bacterial endotoxins on susceptibility to infection with Gram positive and acid fast bacteria. Fed. Proc. 16:856-857.

33. Escomel, E. 1955. Georgraphic localizstion of verruga peruana. La Cron. Med., Lima 52:486-489 and Bull. Soc. Path. Exot. 28:405-409.

34. Fox, H. 1935. Verruga peruana (Carrion's Disease). Based on personal experiences in Peru. J.A.M.A. pp. 985-991.

35. Gastiaburu, J. and R. Rebagliatti. 1909. Sabre la hematologia y la etiologia de la enfermedad de Carrion. Cron. Med., Lima 26:277.

36. Geiman, Q. M. 1941. New media for growth of Bartonella bacilliformis. Proc. Soc. Exptl. Biol. Med. 47:329-332.

37. Giltner, H. A. 1911. Verruca peruana or Carrion's Disease. J.A.M.A. 57:2074-2076.

38. Gomez, M. E. 1914. Epidemiologie del enfermedad de Carrion en las Provincias de Yauyos Canete. Cron. Med., Lima 31:53.

39. Guzman, J. A. 1926. La reaccion de Van den Bergh, hemoaglutinas y hemolisinas en la enfermedad de Carrion. Cron. Med., Lima 43:79-89.

40. Herrer, A. 1953 a. Carrion's Disease. I. Studies on plants claimed to be reservoirs of Bartonella bacilliformis. Am. J. Trop. Med. Hyg. 2:637-643.

41. Herrer, A. 1953 b. Carrion's Disease. II. Presence of Bartonella bacilliformis in the peripheral blood of patients with the benign form. Am. J. Trop. Med. Hyg. 2:645-649.

42. Herrer, A. 1953 c. Carrion's Disease. III. Experimental infection of squirrels. Am. J. Trop. Med. Hyg. 2:650-654.

43. Herrer, A. 1957. Verruga y uta en el valle de Hurillacyan (Dpto. de Ancash). 1. Determiacion de los limites altitudinales de la zona endemica y de la incidencia de ambas enfermedades. Separata de la Revisita de Med. Exptl., Lima 11:40-49.

44. Hertig, M. 1937. Carrion's Disease. V. Studies on phlebotomus as the possible vector. Proc. Soc. Exptl. Biol. Med. 37:598-600.

45. Hertig, M. 1942. Phlebotomus and Carrion's Disease. I. Introduction. Suppl. Am. J. Trop. Med. 22:2-10.

46. Hertig, M. 1942. Phlebotomus and Carrion's Disease. II. Transmission experiments with wild sandflies. Suppl. Am. J. Trop. Med. 22:11-22.

47. Hertig, M. 1942. Phlebotomus and Carrion's Disease. III. Field studies on phlebotomus. Supp. Am. J. Trop. Med. 22:23-59.

48. Hertig, M. 1942. Phlebotomus and Carrion's Disease. IV. Massive infections of the sandfly proboscis with unidentified microorganisms. Suppl. Am. J. Trop. Med. 22:61-81.

49. Howe, C. 1934 a. Immunologic studies in bartonellosis. Arch. Int. Med. 72:147-167.

50. Howe, C. 1943 b. Immune serum therapy in bartonellosis. Arch. Int. Med. 72:429-438.

51. Howe, C. and M. Hertig. 1943. Prophylactic immunization in bartonellosis. J. Immunol. 47:471-482.

52. Howe, C. 1942. Demonstration of agglutinins for Bartonella bacilliformis. J. Exptl. Med. 75:65-75.

53. Jadossohn, J. and G. Seiffert. 1910. Ein fall von verruga peruviana; gelungene uebertragung auf affen. Zeits. f. Hyg. 56:247 (Abstract: Bull. Inst. Pasteur 8:977-978.

54. Jimenez, J. F. 1940. Carrion's Disease. I. Some growth factors for cultivation of Bartonella bacilliformis. Proc. Soc. Exptl. Biol. Med. 45:402-405.

55. Jimenez, J. F. and G. J. Buddingh. 1940. Carrion's Disease. II. Behavior of Bartonella bacilliformis in the developing chick embryo. Proc. Soc. Exptl. Biol. Med. 45:546-551.

56. McKay, D. G., S. J. Merrill, A. E. Weiner, A. T. Hertig and D. E. Reid. 1953. The pathologic anatomy of eclampsia, bilateral renal cortical necrosis, and other acute fatal complications of pregnancy and its possible relationship to the generalized Shwartzman phenomenon. Am. J. Obstet. Gynecol. 66:507-539.

57. Mackehenie, D. and D. Coronado. 1933. Plantas reservorios de virus: Contribution al conocimiento de la fitopatogenesis peruana. Rev. med. Peruana 5:803.

58. Mackie, T. T., G. W. Hunter and C. B. Worth. 1954. A Manual of Tropical Medicine. Saunders Co., Phil. and London. pp. 587-590.

59. Maldonado, A. 1930. Probable role de algunas plantes lactescentes, caracteristica de las quebradas verrucages as y utogenas. Cron. Med., Lima 47:381.

60. Maldonado. 1936. Habitat of verruga peruana or Carrion's disease: Mode of transmission and means of prevention. Arch. Med. Belges (Bull. Internat.) 89:631-638.

61. Manual of Histologic and Special Staining Techniques. Second Edition. McGraw-Hill Book Co., Inc., Blakiston Division. New York-Toronto-London.

62. Mascoli, C. C. and R. G. Burrell. 1965. Preparation of chick embryo cell suspension. pp. 18-19. In: Experimental Virology. Burgess Pub. Co., Minneapolis.

63. Merino, C. 1945. La penicillina en el tratamiento de la enfermedad de Carrion. Rev. med. Peruana 18:329-343.

64. Mitchell, P. D. and R. G. Burrell. 1964. Serology of the Mima-Herellea group and the genus Moraxells. J. Bacteriol. 87:900-909.

65. Monge, C. 1915. Las ictericias hemoliticas en patologia tropical. Rev. Med., Lima 1:5.

66. Moore, G. B., J. M. Puglisevich and S. T. Munoz. 1947. Accion in vitro de la estreptomicina sobre la Bartonella bacilliformis. Arch. Peruanos Patologia Clin. 1:663-670.

67. Noguchi, H. and T. S. Battistini. 1926. Etiology of Oroya fever. I. Cultivation of Bartonella bacilliformis. J. Exptl. Med. 43:851-864.

68. Noguchi, H. 1926 a. Etiology of Oroya fever. III. The behavior of Bartonella bacilliformis in Macacus Rhesus. J. Exptl. Med. 44:697-713.

69. Noguchi, H. 1926 b. Etiology of Oroya fever. VII. The response of the skin of Macacus Rhesus and anthropoid apes to inoculation with Bartonella bacilliformis. J. Exptl. Med. 45:455-460.

70. Noguchi, H. 1927 a. The etiology of verruga peruana. J. Exptl. Med. 45:175-189.

71. Noguchi, H. 1927 b. Etiology of Oroya fever. VI. Pathological changes observed in animals infected with Bartonella bacilliformis. The distribution of parasites in the tissues. J. Exptl. Med. 45:437-454.

72. Noguchi, H. 1928. Etiology of Oroya fever. X. Comparative studies of different strains of Bartonella, with special reference to the relationship between the clinical types of Carrion's disease and the virulence of the infecting organism. J. Exptl. Med. 47:219-234.

73. Noguchi, H., R. C. Shannon, E. B. Tilden and J. R. Tyler. 1929. Etiology of Oroya fever. XIV. The insect vectors of Carrion's disease. J. Exptl. Med. 49:993-1008.

74. Noguchi, H., H. R. Muller, E. B. Tilden and J. R. Tyler. 1929. Etiology of Oroya fever. XVI. Verruga in the dog and the donkey. J. Exptl. Med. 50:455-461.

75. Osborn, J. J., S. M. Rosen, L. Rothfield and B. L. Horecker. 1962. Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of Salmonella. Proc. Natl. Acad. Sci. 48:1831-1838.

76. Ouchterlony, O. 1948. In vitro method for testing the toxin producing capacity of diptheria bacteria. Acta. Pathol. et Mikrobial. Scand. 25:187-191.

77. Payne, E. H. and O. B. Urteaga. 1951. Carrion's disease treated with chloromycetin (Chloramphenicol). Antibiot. Chemother. 1:92-99.

78. Perez Alva, S. 1957. La maladie de Carrion: Essai d'infection experimentale de la souris blanche par voie intranasale par Bartonella bacilliformis. Bull. Soc. Path. Exot., Paris 50:180-188. (Abstract: Trop. Dis. Bull. 54:1172).

79. Peters, D. and R. Wigand. 1955. Bartonellaceae. Bacteriol. Rev. 19:150-159.

80. Peters, D. and R. Wigand. 1952. Neue untersuchungen uber Bartonella bacilliformis. 1. Mitteilung: morphologie der kulturform. Ztschr. Tropenmed. Parasitol. 3:313-326.

81. Peters, D. and R. Wigand. 1953. Enzymatisch-elektronenoptische analyse der nudeinsaureverteil-ung, dargestellt an Escherichia coli als modell. Z. Naturforsch 86:180-192.

82. Peters, D. and R. Wigand. 1954. Lichtund elektronenoptische untersuchungen uber den abban gramnegativer kokken met nucleasen and proteasen. Z. Naturforsch 96:586-596.

83. Pinkerton, H. and D. Weinman. 1937 a. Carrion's Disease. I. Behavior of the etiologic agent within cells growing or surviving in vitro. Proc. Soc. Exptl. Biol. Med. 37:587-590.

84. Pinkerton, H. and D. Weinman. 1937 b. Carrion's Disease. II. Comparative morphology of the etiologic agent in Oroya fever and verruga peruana. Proc. Soc. Exptl. Biol. Med. 37:591-593.

85. Pirsch, J. E., L. A. Mika and M. J. Van der Maaten. 1957. Hypersensitivity of Coxiella burnetii infected guinea pigs to subsequent injections of bacterial endotoxins. Proc. Soc. Exptl. Biol. Med. 96:376-380.

86. Pomerat, C. M. 1946. A review of recent developments on reticula-endothelial immune serum (REIS). The Quart. of Phi Beta Pi. 42:4, 203-208.

87. Reese, J. D., M. E. Morrison and E. M. Fowler. 1950. Complement fixation and Weil-Felix reaction in rabbits inoculated with Bartonella bacilliformis. J. Immunol. 65:355-358.

88. Reynafarje, C. and J. Ramos. 1959. Longevity of the erythrocytes in the anemia of Carrion's disease. An. Fac. Med., Lima 42:9-21.

89. Reynafarje, C. and J. Ramos. 1960. Physiopathology of the anemia of Carrion's disease. An. Fac. Med., Lima 43:501-543.

90. Reynafarje, C. and J. Ramos. 1961. The hemolytic anemia of human bartonellosis. Blood 17:562-578.

91. Ribeyro, R., D. Mackehenie and J. Arce. 1914. Inoculabidad de la verruga peruana a los animales. La Cron. Med., Lima 31:109-121.

92. Ribi, E., K. C. Milner and T. D. Perrine. 1959. Endotoxic and antigenic fractions from the cell wall of Salmonella enteritidis. Methods for separation and some biologic activities. J. Immunol. 82:75-84.

93. Ribi, E., B. H. Hoyer, K. C. Milner, T. D. Perrine, C. L. Larson and G. Goode. 1960. Physical and chemical analysis of endotoxin from Salmonella enteritidis. J. Immunol. 84:32-47.

94. Ricketts, W. E. 1947. Study of incubation period in 13 cases. Am. J. Trop. Med. 27:657-659.

95. Ricketts, E. W. 1948 a. Bartonella bacilliformis anemia (Oroya fever): A study of 30 cases. Blood 3:1025-1047.

96. Ricketts, W. E. 1948 b. Intercurrent infections of Carrion's disease observed in Peru. Am. J. Trop. Med. 28:437-451.

97. Ricketts, W. E. 1949 a. Study of 22 cases with intercurrent malaria. Am. J. Med. Sci. 218:525-530.

98. Ricketts, E. W. 1949 b. Clinical manifestations of Carrion's disease. Arch. Int. Med. 84:751-781.

99. Ristorcelli, A. and Ty Dao van. 1941. Phlebotomus of region of Colombia where verruca peruviana has been endemic for 203 years. Ann. de Parasitol. 18:251-269.

100. Rozeboom, L. E. 1947. The identity of the phlebotomus associated with bartonellosis in Colombia. Ann. Entomol. Soc. Am., Colombus 40:705-714.

101. Salk, J. E. 1944. A simplified procedure for titrating hemagglutinating capacity of Influenza virus and the corresponding antibody. J. Immunol. 49:87-98.

102 Samper, B. and J. A. Montoya C. 1940. Bacteriologic and experimental study of bacteria isolated in epidemic bartonellosis in department of Narino, Colombia. Rev. Fac. de Med., Bogota 9:197-218. ----- (Estudios bacteriologicos y experimentales de un german aislado (en una epidemia de bartonellosis en el departmento de Narino (Colombia)

103. Shannon, R. C. 1929. Entomological investigations in connection with Carrion's disease. Am. J. Hyg. 10:78-111.

104. Spink, W. W. 1954. The significance of endotoxin in Brucellosis: Experimental and clinical studies. Tr. A. Am. Physicians 67:283.

105. Spink, W. W. 1957. The significance of bacterial hypersensitivity in human Brucellosis: Studies on infection due to strain 19 Brucella abortus. Ann. Int. Med. 47:861-874.

106. Spink, W. W. and D. Anderson. 1954. Experimental studies on the significance of endotoxin in the pathogenesis of Brucellosis. J. Clin. Invest. 33:540-548.

107. Strong, R. P., E. E. Tyzzer, C. T. Brues, A. W. Sellards and J. C. Gastiaburu. 1913. Verruga peruviana, Oroya fever and Uts. Preliminary report of the first expedition to South America from the Department of Tropical Medicine of Harvard University. J.A.M.A. 61:1713-1716.

108. Strong, R. P., E. E. Tyzzer, C. T. Brues, A. W. Sellards and J. C. Gastiaburu. 1915. The Harvard School of Tropical Medicine. Report of the first expedition to South America, 1913. Cambridge, Mass.

109. Suter, E. 1962 a. Hyperreactivity to endotoxin in infection. Trans. N.Y. Acad. Sci. II 24:281-290.

110. Suter, E. 1962 b. Hyperreactivity to endotoxin in mice infected with BCG. Studies on the role of concomitant infection. J. Immunol. 89:377-381.

111. Suter, E. 1964 a. Hyperreactivity to endotoxin after infection with BCG. Studies on its distinguishing properties. J. Immunol. 92:49-54.

112. Suter, E. and J. J. Munoz. 1963. Effect of BCG infection on sensitivity of mice to histamine, serotonine and passively induced anaphylaxis. Proc. Soc. Exptl. Biol. Med. 114:211-213.

113. Tamayo, O. 1899. Inoculabilidad de la verruga. Resultado positivo de una inoculacion de sangre verrucose. La Cron. Med., Lima 16:81-84.

114. Thomas, L. and R. A. Good. 1952. Studies on the generalized Shwartzman reaction. I. General observations concerning the phenomenon. J. Exptl. Med. 96:605-624.

115. Thomas, L., F. W. Denny, Jr. and J. Floyd. 1953. Studies on the generalized Shwartzman reaction. III. Lesions of the myocardium and coronary arteries accompanying the reaction in rabbits prepared by infection with group A Streptococci. J. Exptl. Med. 97:751-766.

116. Townsend, C. H. T. 1913 a. The possible and probable etiology and transmission of verruga fever. J. Econ. Ent. 6:211-225.

117. Townsend, C. H. T. 1913 b. La titira es trasmisora de la verruga. La Cron. Med. 30:210.

118. Towsend, C. H. T. 1913 c. A phlebotomus the practically certain carrier of verruga. Science N.S. 38:194-195.

119. Townsend, C. H. T. 1913 d. Progress in the study of verruga transmission by bloodsuckers. Bull. Entomol. Res. 4:125-128.

120. Townsend, C. H. T. 1913 e. The ghost of verruga. Peru Today, Lima 5:840-842.

121. Townsend, C. H. T. 1913 f. Preliminary characterization of the vector of verruga, Phlebotomus verrucarum, new species. Insc. Inscit. Mens. 1:107.

122. Townsend, C. H. T. 1913 g. The transmission of verruga by Phlebotomus. J.A.M.A. 61:1717.

123. Townsend, C. H. T. 1914. The conquest of verruga: A brief statement of the results of the investigations. Peru Today 6:57-67.

124. Townsend, C. H. T. 1915. Identification of the stages in the asexual cycle of Bartonella bacilliformis, the pathogenic organism of verruga, and their bearing on the etiology and unity of the disease. J. Wash. Acad. Sci. 5:662-667.

125. Townsend, C. H. T. 1927. The history, etiology and transmission of peruvian verruga, with an outline of the asexual cycle of its causative organism. West Coast Leader, Lima 15: March 8, 4 page Suppl.

126. Tupper, F. P. 1878-1879. Ueber die verruca peruviana. Berline, g. Schode; Rev. Med. de Chile, pp. 271, 293, 300 and 353.

127. Weinman, D. and H. Pinkerton. 1937 a. Carrion's Disease. IV. Natural sources of Bartonella in the endemic zone. Proc. Soc. Exptl. Biol. Med. 37:596-598.

128. Weinman, D. and H. Pinkerton. 1937 b. Carrion's Disease. III. Experimental production in animals. Proc. Soc. Exptl. Biol. Med. 37:594-595.

129. Weinman, D. 1944. Infectious anemias due to Bartonella and related cell parasites. Trans. Am. Phil. Soc. 33:243-350.

130. Weinman, D. 1946. Human Bartonella infection and African sleeping sickness (field and laboratory experiences). Bull. N.Y. Acad. Med. 22:647-670.

131. Weiss, P. 1927. Hacia una conception de la verruga peruana. Tesis, Facultad de Medicina, Lima.

132. Weiss, P. 1952. Epidemiology of diseases transmitted by Phlebotomus in Peruvian Andes; verruca peruviana, pathogenesis. Rev. Med. Hosp. Obrero. 1:123-134.

133. Wigand, R. and F. Weyer. 1953. Studies of Bartonella bacilliformis transmission to rhesus monkeys and body lice. Ztschr. Tropenmed. u. Parasitol. 4:243-254.

133A. Wilson, G. S. and A. A. Miles. 1961. Topley and Wilson's Principles of Bacteriology and Immunity. Fourth Edition. Williams and Wilkins Co., Baltimore.

134. Wintrobe, M. M. 1956-1958. Clinical Hematology. Fourth Edition, Lea and Febiger, Phil. p. 619.

135. Wintrobe, M. M. 1956-1958. Clinical Hematology. Fourth Edition, Lea and Febiger, Phil. pp. 1128-1129.

136. Zakin, S. J. 1943. Carrion of Carrion's disease, hero and martyr. Quant. Bull. Northwestern Univ. Med. School 17:194-195.

137. Zegarra, A. N. 1955. Bartonellosis and tetracycline. Antibiotic Med. New York 1:201-209. (Abstract: Trop. Dis. Bull. 54:946-947).

138. Zinsser, H., H. Wei and F. Fitzpatrick. 1938. Further studies of agar-slant tissue cultures of typhus Rickettsiae. Proc. Soc. Exptl. Biol. Med. 38:285-288.

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13. ABSTRACT (U) The genus Bartonella was re-evaluated in terms of characterization and identification. Effort included investigation of serological procedures (fluorescent antibody, immuno-diffusion, and hemagglutination techniques) and morphological, biochemical, and cultural studies. Results obtained explained the lack of universally accepted properties of the organism. Investigations of pathogenicity were directed towards (a) reproducing the various forms of Bartonellosis in various animal species and (b) determining the relative susceptibilities or resistances of the animal species to the organism. The rabbit proved more susceptible than the Rhesus monkey, guinea pig, suckling mouse, or hamster when the intravenous, subcutaneous, intraperitoneal, and intratesticular routes of inoculation were employed. A distinct alteration of the rabbit's normally operative hemopoietic system could be induced. However, a distinct anemia and/or an asymptomatic infectious process could not be induced, presumably due to the comparatively high, specific resistance of the animal. Boiled cell suspensions of the organism sensitized the rabbit to such a degree that anaphylactic shock could be provoked. ~~This evidence~~ led to the isolation of an endotoxic component from the organism, and subsequent evidence indicated that this metabolite was responsible for the pathological effects observed in the rabbit. ~~A~~ series of immuno-pathogenicity studies were conducted to elucidate these findings. Sensitization with viable cells of B. bacilliformis increased the susceptibility of rabbits to the lethality of subsequently administered Bartonella metabolites. A distinct immunological basis for the observed lethality was established and is discussed in relation to human Bartonellosis.

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FINAL PROGRESS REPORT

STUDIES ON THE PATHOGENICITY AND RELATED CHARACTERISTICS OF BARTONELLA BACILLIFORMIS*

from

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*In conducting the research reported herein, the investigator(s) adhered to 'Guide for Laboratory Animal Facilities and Care' established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, NAS-NRC.

INTRODUCTION

The investigations to be described were initiated on January 1, 1963 and terminated on April 30, 1966. The work was supported by Contract DA-18-064-AMC-78(A) from the United States Army Biological Laboratories.

To our knowledge, this was the only investigation being carried out in the United States concerning any facet directly related to *B. bacilliformis*. Due to this aspect, we relied on the available literature and correspondence as guides in our early studies. However, certain difficulties were encountered, particularly in the process of developing rapid and efficient methods of cultivation and identification, which necessitated our obtaining first-hand information from Peruvian investigators. Such information, based on our personal experiences in Peru, enabled us to pursue our investigations from the standpoint of validity and will be discussed relative to our investigations in the Discussion portion of the paper.